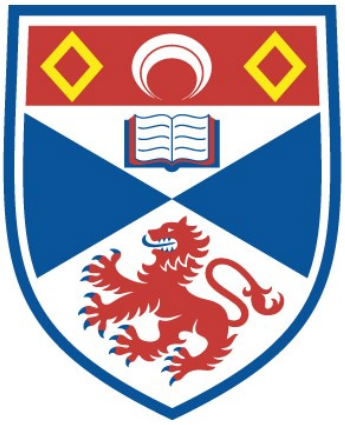
STUDIES ON AMYLOGLUCOSIDASE FROM RHIZOPUS DELEMAR

Luiz Bezerra de Carvalho Junior

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1974

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being a thesis presented by

LUIZ BEZERRA DE CARVALHO JUNIOR

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Biochemistry Department,
The University,
ST. ANDREWS.

December, 1974.

A B S T R A C T

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Amyloglucosidase has been obtained from Rhizopus delemar and purified by ammonium sulphate fractionation; ethanolic precipitation; chromatography on DEAE-cellulose, DEAE-sephadex A-50 and Sephadex G-50/ Amberlite IRC-50. Ethanolic precipitation and chromatography on DEAE-cellulose by stepwise elution have been proved to be the most simple and rapid procedure to remove traces of α-amylase from amyloglucosidase preparations. Enzyme preparations so obtained have been shown to be incapabl of releasing coloured products from Remazol Brilliant Blue derivatives of α-1,4-glucans although they can liberate a limited amount of glucose. In the same way as Cibachron Blue Amylose, Remazol Brilliant Blue derivatives of α-1,4-glucans have been proved to be suitable substrates for the detection of traces of α-amylase in amyloglucosidase preparations.

The apparent kinetic parameters of amyloglucosidase free of traces of α-amylase has been shown to be altered after inclusion of α-amylase. The increased apparent Km and V for the α-1,4-glucan/ amyloglucosidase/α-amylase system compared with those parameters for the system in the absence of α-amylase have been explained on the basis of the decrease in the size of the substrate molecule, which is caused by the action α-amylase(endo-attack pattern).

The apparent kinetic parameters of amyloglucosidase free of traces of α-amylase has also been shown to be altered when partially oxidised α-1,4-glucans are used as substrates., Amyloglucosidase action has been established to be unable of bypassing oxidation points introduced in the α-1,4-glucan by periodate oxidation. A theory has been developed to describe the action of amyloglucosidase on partially oxidised amyloses, which predicts the decrease in the apparent Km and V with the increase in the degre of oxidation of the amylose, as it has been demonstrated experimentally.

A direct recording method of assaying amyloglucosidase has been developed involving ascorbic acid as the oxygen acceptor in a glucose oxidase/peroxidase assay system for glucose. This method permits amyloglucosidase activity to be followed by ultraviolet spectrophotometry. The merits and limitations of this procedure have been discussed.

An immobilised cellulose derivative of amyloglucosidase in which the prosthetic group is involved in the covalent linkage between the enzyme and the support has been investigated and compared with the soluble enzyme. The kinetics, pH profile and the thermal stability of this immobilised enzyme and their comparison with these characteristics for the free enzyme have been investigated.

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. W.M. Ledingham.

C E R T I F I C A T E

I hereby certify that Luiz Bezerra de Carvalho Junior has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

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ACADEMIC RECORD

I matriculated at the Faculty of Medicine of the Universidade Federal de Pernambuco, Brazil, in February 1966, and graduated with the degree of Doctor of Medicine (M.D.) in December 1971.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in January 1972.

A C K N O W L E D G E M E N T S

I should like to express my gratitude to Dr. Ledingham for his continued encouragement and advice throughout the course of this work. I am also thankful to my wife for her incentive and help.

I am grateful for financial support to the Maitland-Ramsay Trust and to the Universidade Federal de Pernambuco.

STUDIES ON AMYLOGLUCOSIDASE FROM RHIZOPUS DELEMAR

I N D E X

ABBREVIATIONS & SYMBOLS

The abbreviations used in this thesis were:

Amgase : - Amyloglucosidase.

CMI : - Commonwealth Mycological Institute.

$\overline{DP}$: - Degree of polymerisation.

DNSA : - 3,5 - Dinitrosalycilic acid.

PAB-cellulose : - p-Aminobenzylcellulose.

PC : - Phosphate/citrate.

PCO : - Phosphate/citrate containing oxalate.

PNPG : - p-Nitrophenyl-α-D-glucoside.

RBB : - Remazol Brilliant Blue.

Other symbols used in this thesis were those recommended by The Biochemical Journal (1).

The citation of bibliographic references followed the recommendations of the IUB Commission of Editors of Biochemical Journal (CEBJ) (2) according to the Numbering System.

1 - INTRODUCTION.

1.1 - GENERAL INTRODUCTION.

Amyloglucosidase was first reported by Corman & Langlyke (3) as a glucose-producing amylase synthesized by the mold Rhizopus delemar.

Because of its ability to convert starch almost quantitatively into D-glucose in recent years amyloglucosidase has assumed considerable industrial importance. On the other hand, special features of its action mechanism have generated considerable academic interest.

There has been great confusion about the trivial nomenclature of amyloglucosidase in the literature, since it is variously reported as amyloglucosidase, glucoamylase, γ-amylase, lysosomal α-glucosidase, exo-1,4-α-glucosidase or acid maltase.

However, the name recommended by the Enzyme Commission is exo-1,4-α-glucosidase whereas the systematic name is 1,4-α-D-glucan glucohydrolase (3.2.1.3) (4). Nevertheless, amyloglucosidase is the commonly used name and will be used in this thesis.

Amyloglucosidase from Rhizopus delemar is capable of splitting α-1,4 bonds (α-1,4-glucanase activity) from non-reducing ends of α-1,4-glucan chains releasing glucose as the sole product. Some amyloglucosidases, including that from Rhizopus delemar, also exhibit α-1,6-glucanase activity.

The following table depicts the major important differences between amyloglucosidase and the two other classes of amylases; α-amylase and β-amylase.

Table 1.1.1. – SIMILARITIES AND DIFFERENCES OF AMYLASES*

CHARACTERISTIC	α-AMYLASE	β-AMYLASE	AMGASE
CLEAVAGE POINT	α-1,4-GLUCOSIDIC BOND	α-1,4-GLUCOSID. BOND	α-1,4- AND α-1,6-@ GLUCOSIDIC BONDS
CONFIGURATION OF NEW REDUCING UNIT	α	β	β
MECHANISM	ENDO-ATTACK	EXO-ATTACK	EXO-ATTACK
END PRODUCTS	OLIGOSACCHARIDES MIXTURE	MALTOSE	GLUCOSE
ACTION AT BRANCH POINT	CAN BYPASS	CANNOT BYPASS	CAN BYPASS
ORIGIN	PLANTS, ANIMALS, MICRO-ORGANISMS	PLANTS, BACILLUS POLYMYXA(6)	ANIMALS, MICRO-ORGANISMS JAPANESE RADISH ROOT(7

* – MODIFIED AFTER ROBYT AND WHELAN(5)

@ – EX. RHIZOPUS DELEMAR.

In the same way as β-amylase, amyloglucosidase inverts the configuration of the product released and acts according to an exo-attack pattern (See Fig. 1.1.1).

Amyloglucosidase has been found in animal tissues, but fungal amyloglucosidases have attracted most interest

Amyloglucosidase has been obtained from Rhizopus delemar (8,9,10) Aspergillus niger (11, 12), Coniophora cerebella (13), Endomycopsis capsularis (14) and Schizophyllum commune (15). Numerous other members of the genus Rhizopus and genus Aspergillus are also known as producers of amyloglucosidases.

A further criteria to differentiate amyloglucosidases from α-glucosidases have been proposed by Reese et al. (16), of which the most important characteristics are:

i) The rate of hydrolysis of oligosaccharides is proportional to the size of substrate for amyloglucosidases whereas the reverse is true for α-glucosidases.

ii) Amyloglucosidases and α-glucosidases invert and retain respectively the configuration of the product released.

The relevant properties of amyloglucosidases, with particular attention to that from Rhizopus delemar, are as follows:

Amyloglucosidase ex. Rhizopus delemar has been reported to have a molecular weight of 70,000 (17), with $E_{1\ cm}^{1\%} = 14.5$ at 280 nm. Pazur (9), however, reported a value of about 100,000. Rhizopus delemar amyloglucosidase exists as a single protein molecule (9).

Amyloglucosidases from fungi are presently considered to be glycoproteins. Pazur (18) has published the aminoacid composition of Rhizopus delemar amyloglucosidase and reports the presence of the following carbohydrate moieties:

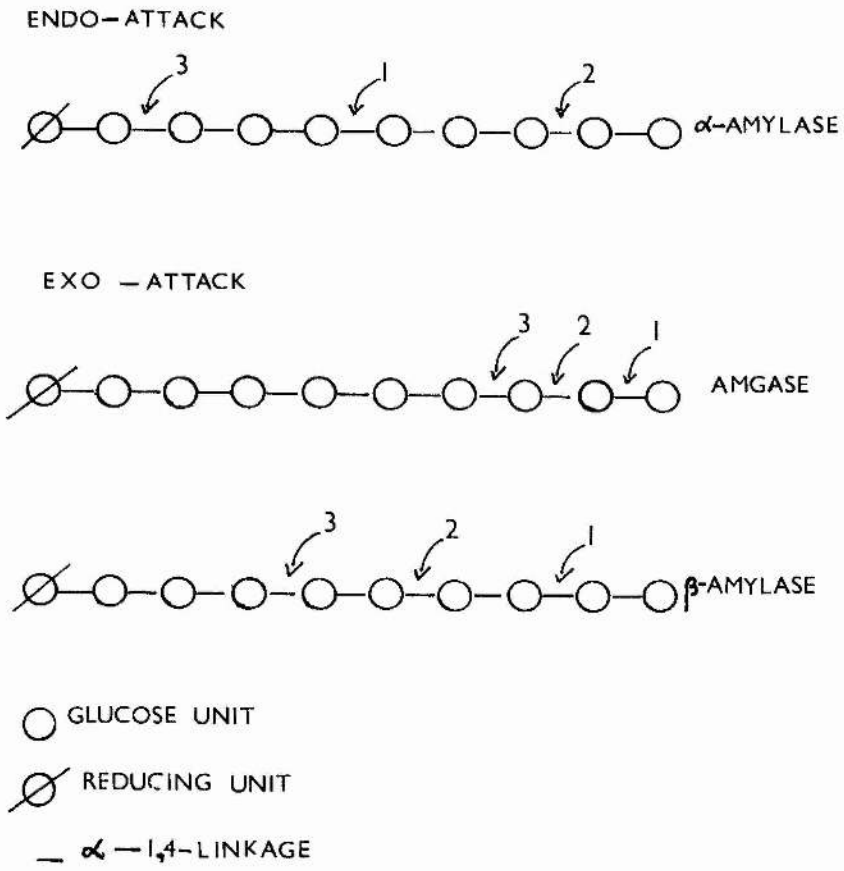


Fig. 1.1.1. Illustration of endo-and exo-attack pattern.

Carbohydrate moiety	residues per molecule of glycoprotein.
Mannose	67
2-amino-2-deoxy-D-glucose (glucosamine)	20

Pazur et al. (19) have also established that exhaustive oxidation of these carbohydrate residues by periodate markedly affects the thermal stability of the enzyme. These authors reasoned that the carbohydrate prosthetic group plays a role in stabilising the tertiary structure of the enzyme. Nakamura & Hayashi (20), studying glucose oxidase from Aspergillus niger (also a glycoprotein), found, after exhaustive oxidation, a similar reduction in thermal stability of the enzyme without any evidence for changes in tertiary structure.

Mylar (21) has also suggested that the neutral character of the carbohydrate moiety aids the transport of the enzyme through the cell membrane of the fungus. Protection for this extracellular enzyme against protease activity could be provided by the presence of these carbohydrates which cause steric hindrance for the close approach of the active centre (22).

Glucosidic bonds seem to link these carbohydrates to threonine and serine of the apoenzyme (23).

Panose (α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1-1,4-D-glucose) and isomaltose (α-D-glucopyranosyl-1,6-D-glucose) are substrates for Rhizopus delemar amyloglucosidase action which catalyses the hydrolysis of both α-1,4 and α-1,6 linkages at the same active centre (24, 25). In the former substrate, the α-1,6 bond is split before α-1,4 bond (26).

Phenyl-α-maltoside is also hydrolysed by amyloglucosidase releasing first glucose and phenyl-α-glucoside, the latter being then split into phenol and glucose (27).

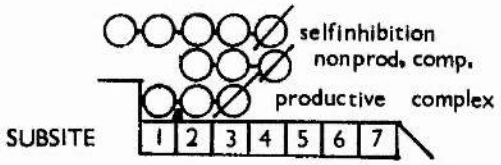
Hollo et al. (28) have suggested that an important role is played by the hydroxyl group on C_3 of the pyranose ring in the formation of amyloglucosidase-substrate complex. This assumption is based upon inhibition studies with glucose derivatives on amyloglucosidase activity. See table below.

Inhibitor	Type of inhibition	Ki(M/l)	Affinity (1/Ki)
α-D-glucose	competitive	4.5×10^{-3}	220.0
2-Deoxy-α-D-glucose	competitive	5.0×10^{-3}	200.0
6-Deoxy-α-D-glucose	competitive	5.5×10^{-3}	182.0
3-o-Methyl-α-D-glucose	non-competitive	5.3×10^{-2}	18.8

Hiromi et al. (17) have proposed a subsite theory for amyloglucosidase in which the active centre of the enzyme is visualised as composed of several subsites each one having different affinities for the glucose residues of the α-1,4-glucan.

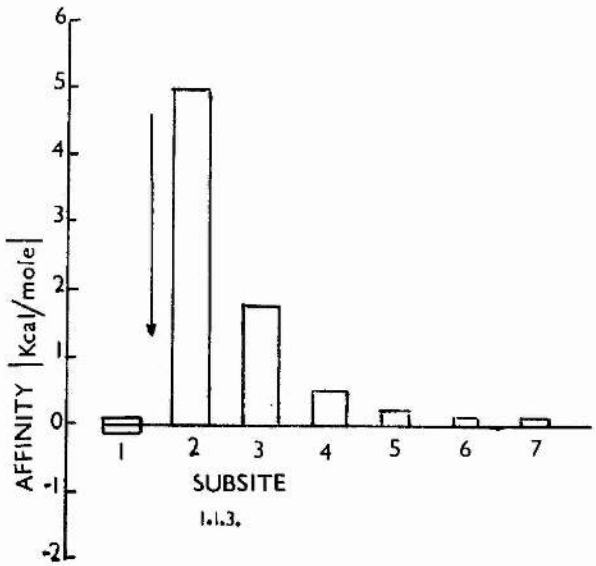
By using substrates with DP varying from 2 to about 15, the affinities of seven subsites were established. Subsites were numbered counting from the terminal in which the non-reducing glucose unit is situated in the formation of productive enzyme-substrate complex. The catalytic site lies between subsite 1 and 2. The second subsite has the highest affinity which decreases towards the reducing end.

Fig. 1.1.2 shows a schematic representation of the subsite theory of amyloglucosidase from Rhizopus delemar, whereas Fig. 1.1.3 displays a histogram of the affinity of each subsite expressed in Kcal/mole.



GLUCOSE UNIT
REDUCING UNIT
CATALYTIC RESIDUES

1.1.2.



1.1.3.

Fig. 1.1.2. Schematic representation of subsite theory showing the formation of a productive and non-productive complexes as well as competitive inhibition by internal units of substrate.

Fig. 1.1.3. Histogram showing the affinities of different subsites. The arrow indicates the position of the catalytic site.

The participation of carboxylic groups in the active site of amyloglucosidase was demonstrated by Hiromi et al. (25) and also by Gray & Jolley (29).

The Michaelis constant (Km) and maximal velocity (V) of amyloglucosidases are known to depend on the DP of substrates. Table 1.1.2. shows some values of Km and k_{+2} concerned with amyloglucosidase ex. Rhizopus delemar collected from various authors. The values of k_{+2} are more significant than V since usually the amount of enzyme varies with the experiment ($k_{+2}=V/(E)$).

These results basically show:

i) The Michaelis constant (Km) decreases with the DP of substrate. Its value for an average DP equal to 800 is about 3,000-times lesser than for DP equal to 2 (30).

ii) k_{+2} also appears to follow the same pattern beyond a certain critical value of DP. For instance, the value (14.4 s^{-1}) for amylose with an average DP of 800 is less than 50% of the value when DP is equal to 7 (31 s^{-1}).

iii) Contrary to what is stated above, k_{+2} increases in parallel with DP up to maltoheptaose (17).

Thoma & Koshland (31) have proposed an explanation for the behaviour of Km and k_{+2} of exo-amylases depending on the size of substrates, namely, items i and ii above. They have proposed the possibility of competitive inhibition by the internal residues of the substrate with the enzyme active centre. Therefore, this inhibition will increase with the chain length of the substrate causing a decrease in the apparent Km and apparent maximal velocity. See section 4.3. for fuller discussion.

TABLE 1.1.2. - RATE PARAMETERS FOR RHIZOPUS DELEMAR AMYLOGLUCOSIDASE HYDROLYSIS OF SUBSTRATES WITH DIFFERENT MOLECULAR WEIGHTS.

Substrate	DP*	Km(M)	$k_{+2}(s^{-1})$@	Reference
Maltose	2	1.1×10^{-3}	4.6	17
		1.16×10^{-3}	3.02	30
		6.6×10^{-3}	n.d.	8
Maltotriose	3	3.6×10^{-4}	23	17
		2.02×10^{-4}	12.1	30
Maltotetrose	4	2.5×10^{-4}	33	17
Maltopentose	5	1.6×10^{-4}	32	17
		1.17×10^{-4}	27.7	30
Maltohexaose	6	1.2×10^{-4}	28	17
Maltoheptaose	7	1.1×10^{-4}	31	17
Maltodextrin	15.5&	6.5×10^{-5}	24	17
	15&	4.9×10^{-5}	21	30
Amylose	800&	3.84×10^{-6}	14.4	30
	n.d.	4.4×10^{-6}£	n.d.	8
Amylopectin	n.d.	4.1×10^{-7}£	n.d.	8

* - in glucose units.

@ - Obtained dividing the maximal velocity ($M.min^{-1}$) by the enzyme concentration (M).

& - Average DP

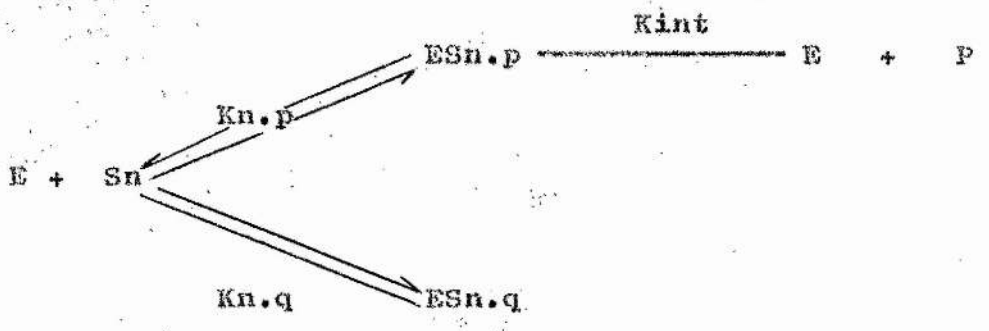
£ - Authors used the values of 1.5×10^{-5} and 1.5×10^{-6} for the mol.wt. of amylase and amylopectin, respectively.

n.d. - not determined.

However, Thoma & Koshland's proposal does not explain item iii (the increase of k_{+2} up to DP equal to 7).

The subsite theory of Hiromi *et al.* (17) provides a satisfactory explanation for item iii. This analysis is as follows:

Suppose the reaction for amyloglucosidase is given by the equation:



Where

E : - Enzyme.

Sn : - Polymer-substrate containing n monomers.

P : - Product.

ESn.p : - Productive complex.

ESn.q : - Non-productive complex.

Kint : - Intrinsic rate constant or "true" k_{+2}.

Kn.p : - Association constant for the productive complex formation.

Kn.q : - Association constant for the non-productive complex formation.

The percentages of productive and non-productive complexes of various linear substrates (DP equal to 2 up to 7) are determined by the affinity required by the subsites as shown in Fig. 1.1.3. Although the "true" k_{+2} is independent on DP, the observed n dependence of k_{+2} arises simply from the multiplicity of binding modes of substrate to the enzyme. Therefore, this effect does not exist for larger substrate

molecules, namely, higher than DP equal to 7. It is interesting to note that this phenomenon is in no way connected with self-inhibition of substrate proposed by Thoma & Koshland (31).

1.2. - THE DETECTION OF α - AMYLASE IN AMYLOGLUCOSIDASE PREPARATIONS.

1.2.1. - THE USE OF PARTIALLY OXIDISED AMYLOSES.

Marshall & Whelan (32) have developed a method to detect traces of α -amylase in preparations of β -amylase or amyloglucosidase. This method involves the use of partially oxidised amylose.

Exo-amylase activity is blocked by the presence of these oxidation points whereas endo-amylase activity can by-pass them. A schematic illustration of these features is given in Fig. 1.2.1.1.

The molecular structure of amylose and the modifications introduced by the oxidation with periodate are presented in Fig. 1.2.1.2.

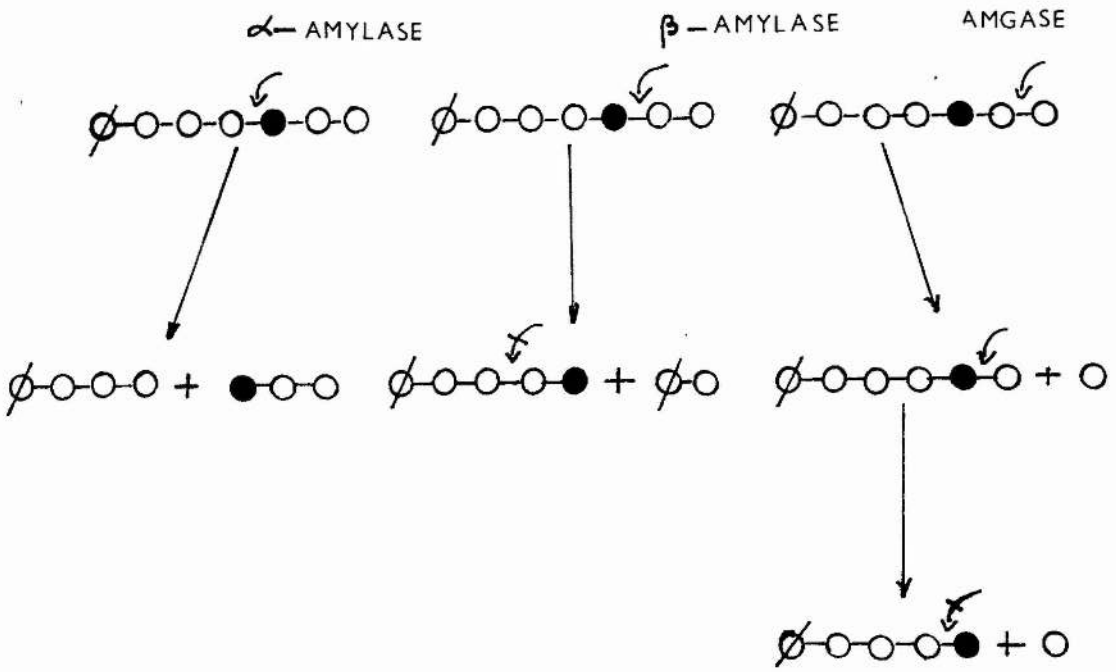
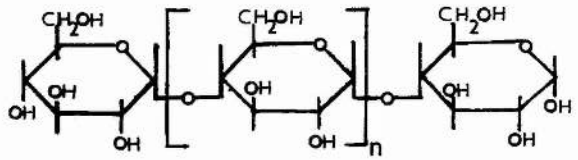
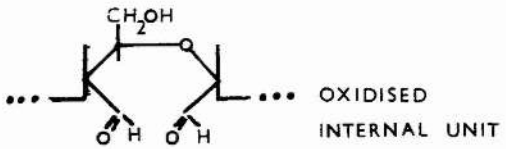


Fig. 1.2.1.1. Schematic mechanism for endo-and exo-amylases on partially oxidised amylose.





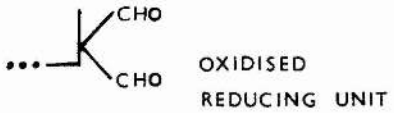


Fig. 1.2.1.2. Molecular structure of amylose and modifications induced by periodate oxidation in its non-reducing, internal and reducing units (glucose).

1.2.2. - THE USE OF CHROMOGENIC SUBSTRATES.

Chromogenic substrates can be defined (33) as substrates in which, following enzyme action, a coloured product is formed either directly or upon the addition of a single or simple reagent.

The introduction of chromogenic substrates has contributed significantly to the detection of traces of endo-amylase activity in exo-amylase preparations.

The use of such substrates in the study of α-1,4-glucanases was first reported by Rinderknecht et al. (34), based on the following principles:

i) Dye molecules are incorporated into α-1,4-glucans by covalent linkage rendering the dyed polysaccharide water insoluble.

ii) This dyed polysaccharide is shaken with the enzyme and the presence of small soluble portions of the chromogenic substrate, containing the dye, released by enzymic hydrolysis are detected spectrophotometrically in the supernatant after centrifugation (See Fig. 1.2.2.1.).

Marshall (35) has shown that amyloglucosidase is not able to release soluble coloured products from the chromogenic substrate CIBACHRON BLUE AMYLOSE whereas α-amylase can do so. This difference in action pattern can be easily interpreted in terms of exo- and endo-attack, in which amyloglucosidase will not "recognise" the non-reducing glucose terminal unit if a chromophore group is attached to it. On the other hand, amyloglucosidase may be able to release a certain number of glucose residues sequentially from a non-reducing chain end depending on the position of attachment of the dye molecule.

Similar arguments will obviously apply to the action pattern of β-amylase on these substrates.

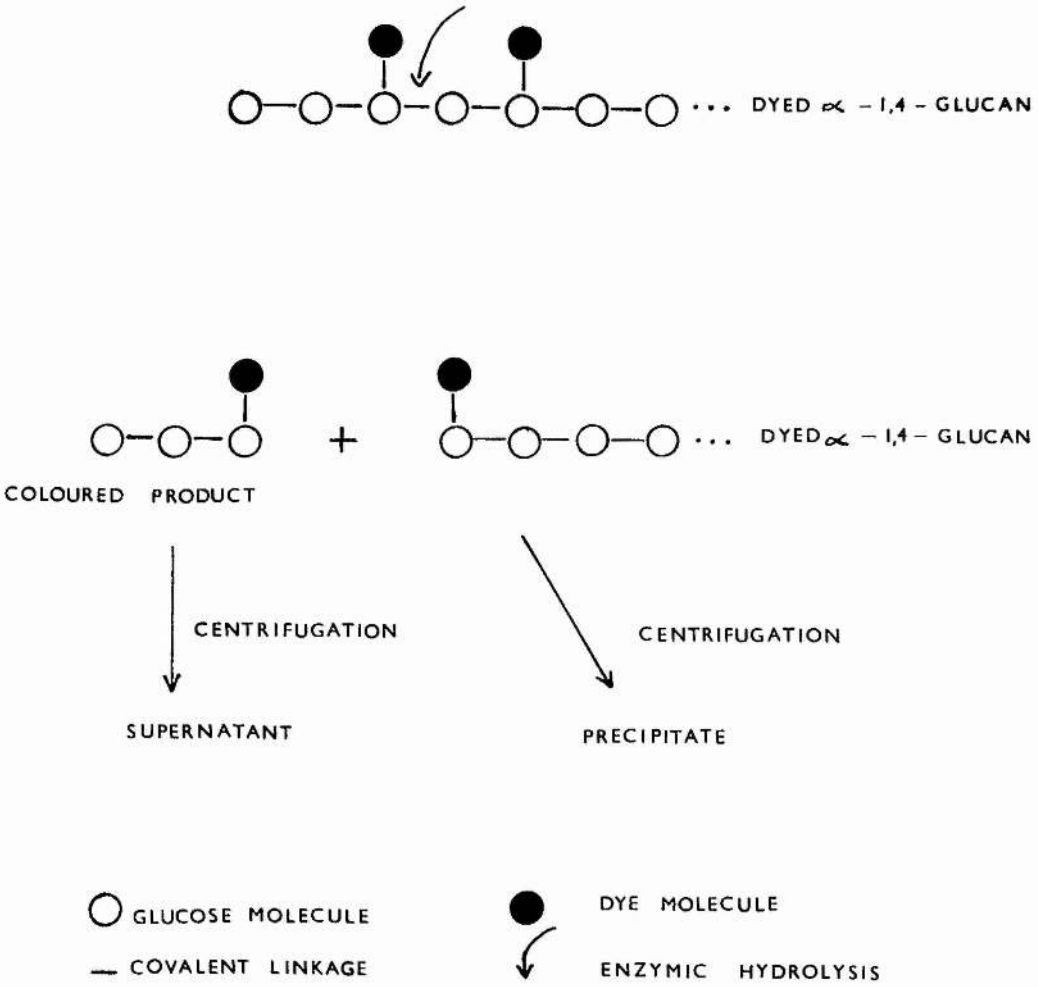


Fig.1.2.2.1. Scheme showing an enzymic hydrolysis of dyed-polysaccharide and the detection of coloured product released.

Exo-attack (amyloglucosidase and β-amylase) on chromogenic substrates is shown in Fig. 1.2.2.2., whereas the endo-attack is presented in previous figure, Fig. 1.2.2.1.

The most commonly used chromogenic substrates for the studies of α-1,4-glucanases belong to two groups: Remazol Briilitant Blue and Cibachron Blue F3GA derivatives of α-1,4-glucans reported by Rinderknecht *et al.* (34) and Klein *et al.*(33), respectively.

The molecular structures of both chromophores and how they are linked to the polysaccharide (amylose) are presented in Fig. 1.2.2.3.

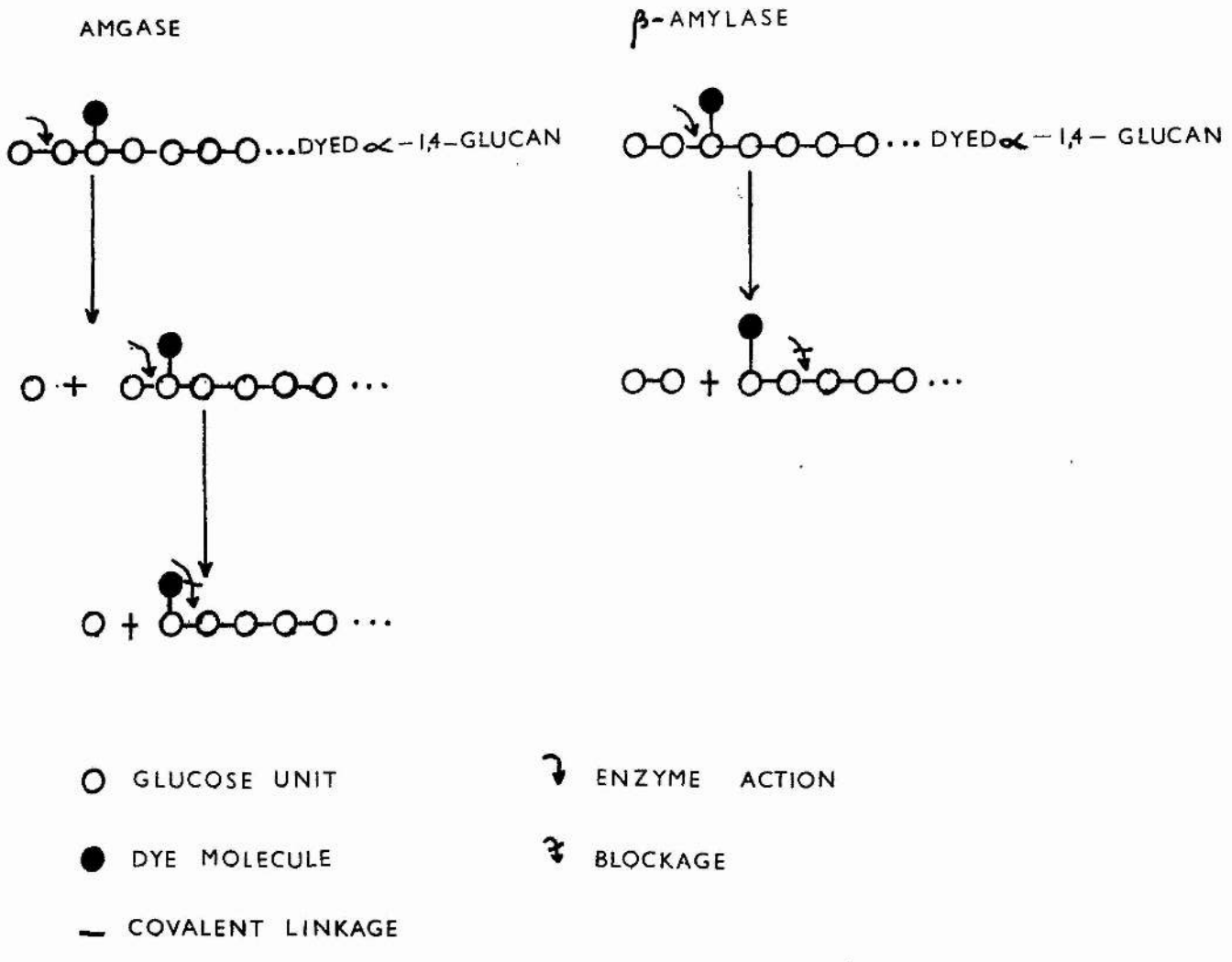
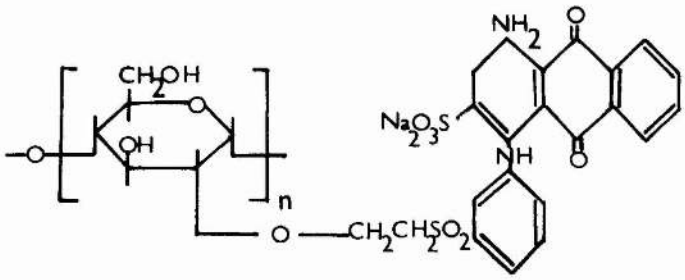
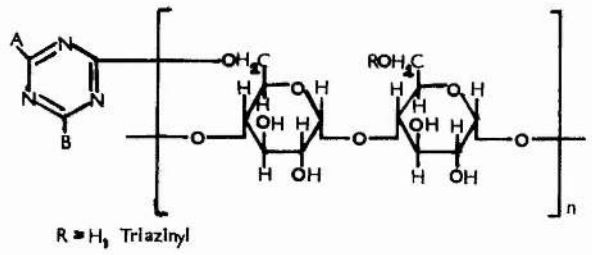


Fig. 1.2.2.3. Enzymic hydrolysis of dyed-polysaccharide according to the exo-attack pattern.



AMYLOSE AZURE



CIBACHRON BLUE — AMYLOSE

Fig. 1.2.2.3. Molecular structures of amylose azure and cibachron blue amylose. The groups in the positions A and B in cibachron blue amylose have not been completely identified.

1.3. - SURVEY OF IMMOBILISED AMYLOGLUCOSIDASE.

There are practical and theoretical reasons for studying enzymes bound to water-insoluble supports and they have been discussed in reviews dealing with immobilised enzyme derivatives (36, 37). The investigation of immobilised amylases is therefore justified on the basis of its economic use in starch liquification and its application in the study of the effect of the microenvironment on the mode of action of enzymes which act on substrates of high molecular weight.

Insoluble derivatives of α-amylase, β-amylase and amyloglucosidase have been prepared by a variety of physical and chemical methods. The present work has been concerned with immobilised amyloglucosidase.

Immobilised preparations of amyloglucosidase have been prepared by:

1 - Chemical coupling to DEAE-cellulose using the bifunctional reagent 2-amino-4,6-dichloro-s-triazine (38, 39)
2 - Chemical coupling to diazotised 3-(p-aminophenoxyl)-2-hydroxypropyl ether of cellulose (40).
3 - Chemical coupling to CM-cellulose azide (41).
4 - Physical coupling to DEAE-cellulose (42).
5 - Chemical coupling to poly(allyl carbonate) (43).
6 - Chemical coupling to CM-cellulose hydrazide (44).
7 - Entrapment in polyacrylamide matrix (45).
8 - Chemical coupling to polystyrene (46).
9 - Chemical coupling to γ-isothiocyanatopropyl-diethoxysilyl glass (47).

Table 1.3.1. shows the percentage activity retained by amyloglucosidase coupled by these procedures.

As shown in table 1.3.1., the enzyme activity retained in the immobilised derivatives varied widely.

TABLE 1.3.1. - PROTEIN COUPLED AND ENZYME ACTIVITY IN THE IMMOBILISED AMYLOGLUCOSIDASE DERIVATIVES.

Method	Bound protein (mg/100mg of derivative)	Enzyme (units/mg of bound protein)	Activity retained after (%)	Reference
Chemical coupling to glass	1	1	66-70	47
Physical coupling to DEAE-cellulose	1-17.5	8.6-28.7*	16.7-55.8	42
Chemical coupling to CM-cellulose hydrazide	23.0	20.39	41.5	44
Chemical coupling to DEAE-cellulose	12.0	1-1.5	20-30	39
Entrapment in poly-acrylamide	n.d.	n.d.	20	45
Diazo-coupling to cellulose	1.25	9.27	17.7	40
Chemical coupling to poly(allyl carbonate)	0.07-0.86	0.45-1.66	1.5-5.4	43
Chemical coupling to polystyrene.	0.02©	n.d.	2.37©	46
Chemical coupling to CM-cellulose azide	n.d.	n.d.	2.2	41

* - The unit defined by the authors was converted to that recommended by the Enzyme Commission and used in this thesis.

© - Calculated from Fig.1 and 2. Higher values caused decrease in the resulting specific activities.

n.d. - not determined.

Flemming et al. (47) have reported the highest recovered specific activity (68%) of immobilised amyloglucosidase at the present time using γ-isothiocyanatopropyl-diethoxysilyl groups containing glass. However, the bound protein for this method was less than those described for physical and chemical coupling to DEAE-cellulose and chemical coupling to CM-cellulose Hydrazide (See Table 1.3.1).

Although the enzyme physically coupled to DEAE-cellulose shows a high retained specific activity, physical coupling suffers from the disadvantage of being dependant on such parameters as the pH and ionic composition of the micro-environment changes which may lead to leaching-off of the enzyme.

Chemical coupling of amyloglucosidase to DEAE-cellulose (39) yielded an immobilised preparation with a retained specific activity of 20-30%.

Christison (44) achieved a reasonable specific activity using an immobilisation technique involving the prosthetic group of the amyloglucosidase. The apoenzyme not being involved in the formation of the covalent linkage between the enzyme and the support, provides a possible explanation for the value of 41.5% specific activity retained by the enzyme after coupling in this case. It has been suggested by several authors (19, 20) that the prosthetic group has no apparent role in the catalytic functions of glycoprotein enzymes such as amyloglucosidase from fungi but could contribute towards the stability of the enzyme. However, Christison has not further investigated the characteristics of his immobilised amyloglucosidase preparation.

Gruesbeck & Rase (45) have reported an entrapped amyloglucosidase in polyacrylamide matrix with 20% of activity retained. The authors have established an optimum polymer

preparation which produced the highest specific activity retention with a minimum leakage of entrapped enzyme. A similar recovery was achieved by Baker et al. (40) (17.7%) covalently coupling amyloglucosidase to a diazotised derivative of cellulose.

Ledingham & Ferreira (46) have described an immobilised amyloglucosidase derivative with a very low retention of activity in which polystyrene beads were used as carriers. The authors themselves acknowledge the disadvantages of using this support since the low ratio for surface area/volume of a solid bead limits the amount of bound protein per weight of a polystyrene bead. Furthermore, Filippusson & Hornby (48) have reported that the hydrophobic micro-environment provided by the polystyrene causes remarkable changes in Km and V (40 mM and 0.0018 μmol. min^{-1}.mg. $protein^{-1}$) of sucrose hydrolysis by polystyrene-supported β-fructofuranosidase compared with those of the free enzyme (17-20mM and 0.016 μmol.min^{-1}.mg $protein^{-1}$).

In addition, the kinetics, pH profiles and the thermal stability of immobilised enzymes and their comparison with these characteristics for free enzymes have been investigated. These investigations have provided a basis for mathematical models describing the action of immobilised enzymes (49, 50).

The interpretation of experimental data from amylases requires caution when heterogeneous substrates are employed. It is now realised that even although two samples of polymer have the same average degree of polymerization they can have different distributions which yield different results upon enzymic hydrolysis (51).

Obviously, the same care is applied to comparisons between results obtained by different authors working with different sources of substrates. These remarks are also valid

for bound amyloglucosidases.

Unfortunately, much information is not available about kinetic parameters of immobilised amyloglucosidase in the literature. Ledingham & Ferreira (46) have observed a tenfold increase in Km compared with the soluble enzyme when polystyrene beads were used as support. Maeda & Suzuki (41) have also reported a slightly increased Km using both maltose (1.74×10^{-3} M compared with 0.96×10^{-3} M for the native enzyme) and amylose (1.24×10^{-7} M compared with 0.74×10^{-7} M for the native enzyme) as substrates for amyloglucosidase immobilised on CM-cellulose azide.

O'Neill *et al.* (39) have related a large decrease in the value of Km using maltose as substrate for amyloglucosidase immobilised on DEAE-cellulose - 1.4 mM for attached amyloglucosidase relative to 6.1 mM for native enzyme. This decrease is not explained either by ionic charge effect (maltose is uncharged) or by diffusion control of the substrate (higher value of Km should be expected). However, these authors (39) have found a decrease in the value of V(1.1 units. mg protein^{-1}) relative to the value for native enzyme (4.7 units. mg protein^{-1}).

Hornby *et al.* (52) have described a mathematical model in which the diffusion of the substrate into the micro-environment of the immobilised enzyme is considered in addition to other factors affecting immobilised enzyme action. Since high molecular weight substrates rather than charged ones are employed in this present case, diffusion control on the rate of substrate penetration plays a more important role than ionic charge effect. If diffusion of the substrate to the active site, which is inversely proportional to its size, is such that at equilibrium the concentration of the substrate is less in the micro-environment of the bound enzyme than that measured in

the bulk of the substrate solution, then a higher value in the observed Km would be expected.

Gruesbeck & Rase (45) have demonstrated that the rates of hydrolysis of maltose and amylose were unaltered following gel inclusion of the enzyme whereas the rate of amylopectin hydrolysis was markedly reduced. Evidence for the exclusion of this latter substrate on account of its size was given.

Diffusion problems of high molecular weight substrates are reported to be much reduced in the case of water-soluble support materials such as Dextran, DEAE-dextran and CM-cellulose, as compared with water-insoluble supports (53). Unfortunately, there exists little kinetic data to date.

In general, immobilisation on charged supports causes shifts in the pH profiles compared with the native enzyme. These shifts have been observed for several enzymes attached to charged supports both insoluble (54) and soluble (53). If the enzyme is bound to a negatively charged matrix, the localised low pH (due to higher concentration of hydrogen ions) in the micro-environment, compared to the pH in the bulk of the solution, will cause an apparent alkaline shift in the pH profile. The reverse applies to an enzyme bound to a positively charged support. However, attachment to uncharged carriers does not alter the optimum pH compared with the free enzyme. Gruesbeck & Rase (45) have demonstrated that for amyloglucosidase entrapped in crosslinked polyacrylamide (uncharged matrix) no alteration in the pH profile has been observed. On the other hand, the pH profile for amyloglucosidase covalently coupled to DEAE-cellulose (39) (positively charged matrix) showed an acidic shift. Moreover, the pH profile for amyloglucosidase ionically bound to DEAE-cellulose also showed a slightly acid shift (optimum pH about 4.0) compared with that known for

free amyloglucosidase (pH 4.5-5.0).

It is generally agreed that immobilised preparations of enzymes are more stable than their free preparations and this extra-stability has industrial advantages.

Immobilised amyloglucosidases which have more stability than their native preparations have been reported by various authors (39, 40, 42, 45).

Ledingham & Ferreira (46) have reported a greater degree of multiple action exhibited by polystyrene-amyloglucosidase compared with the free enzyme, finding a greater liberation of reducing power (glucose) for a given fall in blue value (a measure of average chain length) in the polystyrene-enzyme compared with the free enzyme.

1.4. - THE AIMS OF THIS RESEARCH.

The work presented here was performed with a view to fulfilling the following objectives:

Using amyloglucosidase obtained from *Rhizopus delemar* to establish

1 - An accurate, rapid and direct method for assaying amyloglucosidase.

2 - A purification procedure to remove traces of α-amylase contaminating amyloglucosidase preparations and the employment of such enzyme preparations to investigate

   a) Amyloglucosidase action on Remazol Brilliant Blue derivatives of α-1,4-glucans.

   b) The kinetics of amyloglucosidase in the absence and presence of α-amylase.

   c) The kinetics of amyloglucosidase on partially oxidised amyloses.

3 - A comparison between the kinetics of free amyloglucosidase and immobilised amyloglucosidases in which a) the prosthetic group is involved in the covalent linkage between the enzyme and the support, and b) the apoenzyme is involved in the covalent linkage.

2 - MATERIALS

The chemicals used in this project were obtained from:

1 - BDH Chemicals Ltd., Poole, England.

2 - The Boehringer Corporation Ltd., London, England.

3 - Calbiochem, Los Angeles, U.S.A.

4 - Oxoid Ltd., London, England.

5 - Pharmacia, Uppsala, Sweden.

6 - Sigma London Chemical Co. Ltd., Surrey, England.

7 - Whatman Biochemicals Ltd., Kent, England.

They are listed below in alphabetical order followed by a number which refers to the cited suppliers.

Agar no.3 (4)

Amberlite IRC-50 (1)

p-Aminobenzylcellulose (1)

α-amylase (6)

β-amylase type II - B (6)

Amylopectin Azure A grade (3)

Amylose type I (6)

Amylose Azure B grade (3)

Albumin, bovine (6)

L-Ascorbic acid (1)

Carboxymethylcellulose CM-11 (7)

Carboxymethylcellulose hydrazide (6)

Casein (6)

DEAE-cellulose DE-32 (7)

DEAE-sephadex A-50 (5)

3,5-Dinitrosalycilic acid (1)

Folin Ciocalteu's phenol reagent (1)

Glucose Analar (1)

Glucose oxidase grade I (2)

Glucose oxidase type II (6)

α-glucosidase type I (6)

Hydrazine hydrate (1)

Hydrogen peroxide Analar (1)

Lysozyme grade I (6)

Malt extract (4)

Maltose (6)

Mycological peptone (4)

Neocuproine HCl (6)

p-Nitropenyl- α-D-glucoside (6)

Peroxidase type I (6)

Peroxidase type crude (6)

Sephadex G-50 (5)

Sodium metaperiodate (1)

Starch (soluble) Analar (1)

All other reagents were of analytical grade.

Visible range spectrophotometry was carried out using a Unicam SP 600 spectrophotometer (Unicam Instruments, Cambridge, England).

3. - GENERAL METHODS

3.1. - PREPARATION OF THE ENZYME FROM RHIZOPUS DELEMAR.

Stock cultures:

Stock cultures of the fungus Rhizopus delemar (CMI; 44, 245) were maintained on agar slants in Universal bottles in the following medium:

Medium	% (w/v)
Malt extract	3.0
Mycological peptone	0.5
Agar	1.2
Tap water.	

Enzyme production cultures:

100 ml of the above liquid medium (without agar) was inoculated with a loopful of spores from a stock culture and grown at 30^{o} for 48 h in a New Brunswick Orbital Shaker (submerged culture).

The whole of this culture was used to inoculate 12 l of the same medium in a 14 l New Brunswick Bench Top Fermentor. Growth was continued for 36-48 h at 32^{o}. Aeration was 10 $l.min^{-1}$ and agitation 400 $rev.min^{-1}$.

Supernatant amyloglucosidase activity was assayed by DNSA method 6-hourly after 24 h of growth and the fermentation was stopped when activity started to decline.

Harvesting:

Then the culture was filtered through a coarse Buchner filter to remove fungal material, and reduced in volume to 1 litre by rotary evaporation at 40^{o} under vacuum (approx. 15mmHg). The concentrate was centrifuged at 12,000 g for 10 min and the supernatant freeze-dried. This lyophilised material so obtained was termed crude enzyme preparation from the fermentation of Rhizopus delemar.

3.2. - AMYLOGLUCOSIDASE ASSAY.

Amyloglucosidase activity was measured, throughout this project, by assaying the amount of glucose released in an incubation at 40°. The composition of the incubation medium was, unless otherwise stated, as follows:

1% (w/v) soluble starch in 25 mM sodium phosphate/ citrate buffer, pH 5.0.

An aliquot of the incubation mixture was withdrawn at appropriate time intervals and the glucose content determined by the DNSA method, or the neocuproine method, or Lloyd and Whelan's method (discontinuous assays). These procedures are subsequently referred to as DNSA, neocuproine and Lloyd and Whelan's methods of assaying amyloglucosidase.

Amyloglucosidase action was also followed by continuous assay employing either spectrophotometry (ascorbic acid method) or the oxygen electrode (glucose oxidase/oxygen electrode method).

One unit of activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under the specified conditions.

3.3 - DISCONTINUOUS ASSAY OF AMYLOGLUCOSIDASE - GLUCOSE DETERMINATION.

3.3.1 - REDUCING METHOD USING 3,5-DINITROSALYCILIC ACID (DNSA).

The employement of DNSA to determine reducing sugar was according to the recommendations of Bernfeld (55).

3.3.1.1. - MANUAL METHOD.

Preparation of DNSA reagent:

100 ml of 2 M NaOH was heated to about 70° and DNSA (5g) was slowly added with constant stirring. Upon complete solution of the DNSA, 100 ml of distilled water at approx. 70° was added. Sodium potassium tartrate (150g) was slowly added. When it was dissolved the volume was made up to 500 ml with warm distilled water.

Assay:

A volume of 2.0 ml DNSA reagent was added to 1-5 ml of sugar solution (containing a maximum of 1.8 mg glucose or equivalent). The tubes were capped and incubated in a vigorously boiling water bath for 5 min and then cooled. The solutions were diluted to 20 ml and the absorbance was measured at 540 nm against a blank similarly processed.

A calibration curve for glucose is shown in Fig. 3.3.1.1.1.

3.3.1.2. - AUTOMATED METHOD

An automated method, based on the Technicon flow principle (Technicon Corp., U.S.A.), for the determination of sugar by the DNSA method was used and its flow diagram is shown in Fig. 3.3.1.2.1.

Fig. 3.3.1.2.2. shows the calibration curve for glucose using the DNSA-automated procedure.

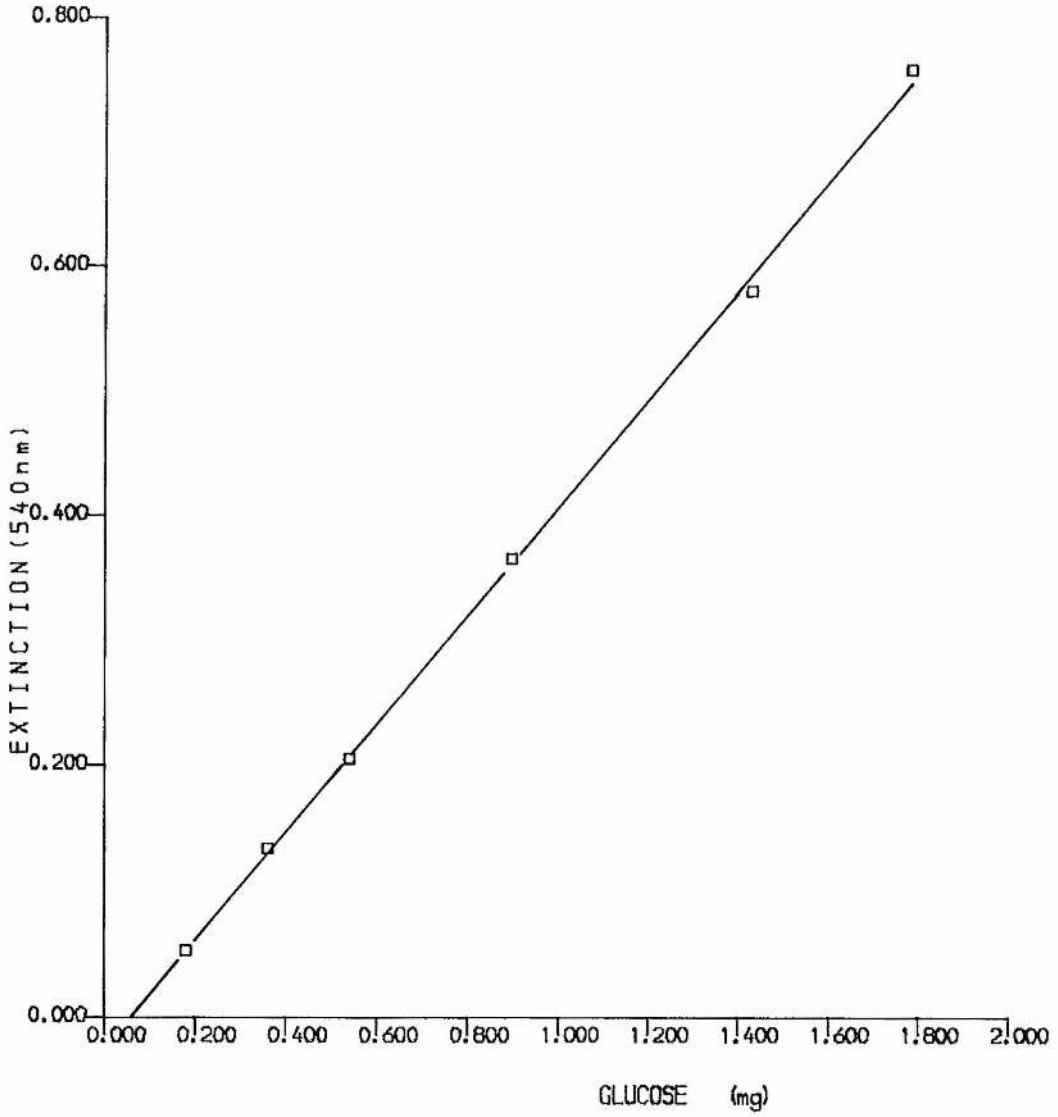


Fig. 3.3.1.1.1. Standard curve for glucose determination using the manual DNSA method.

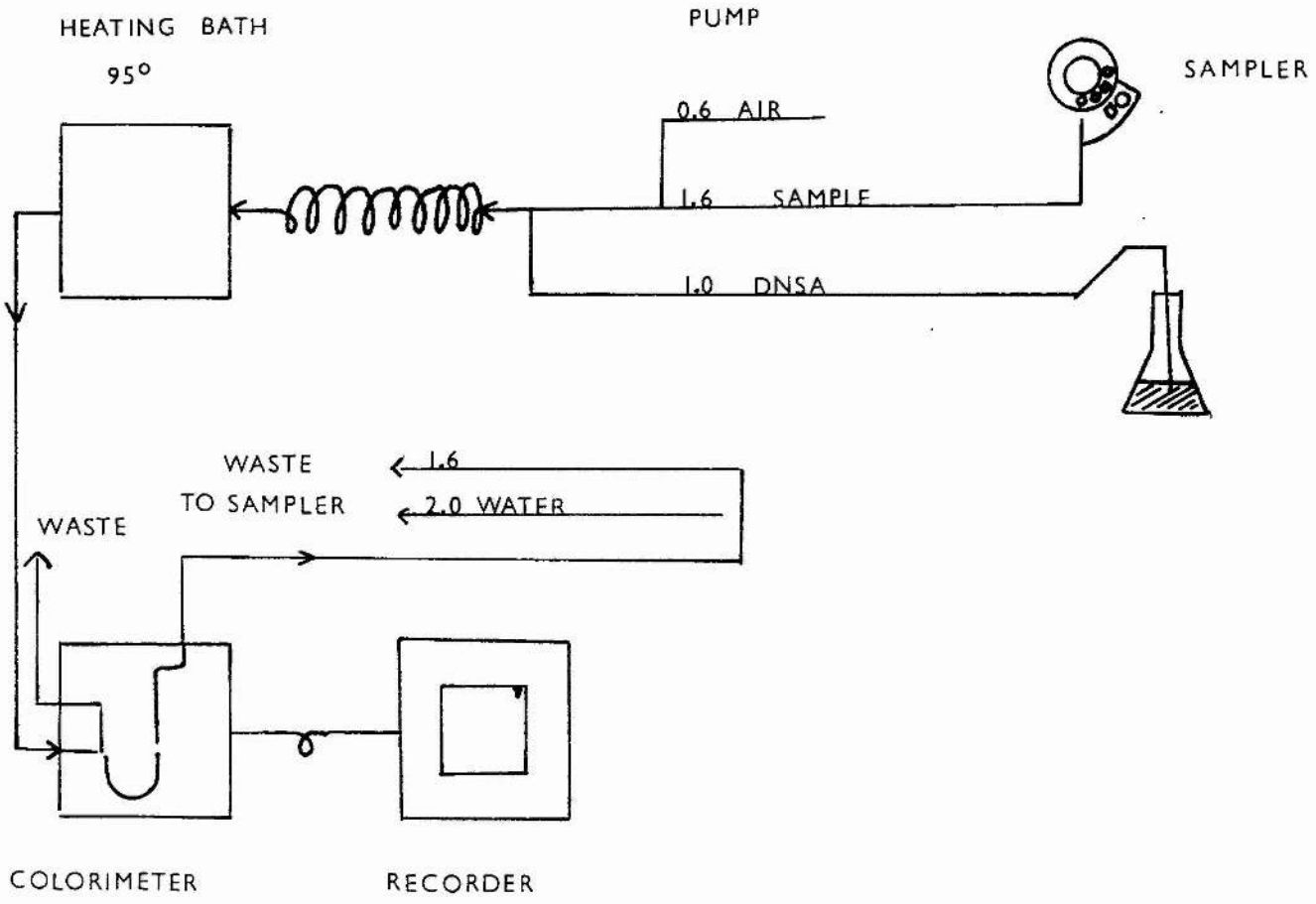


Fig. 3.3.1.2.1. Flow diagram for the automated glucose determination using the DNSA method. A Technicon analyzer was employed.

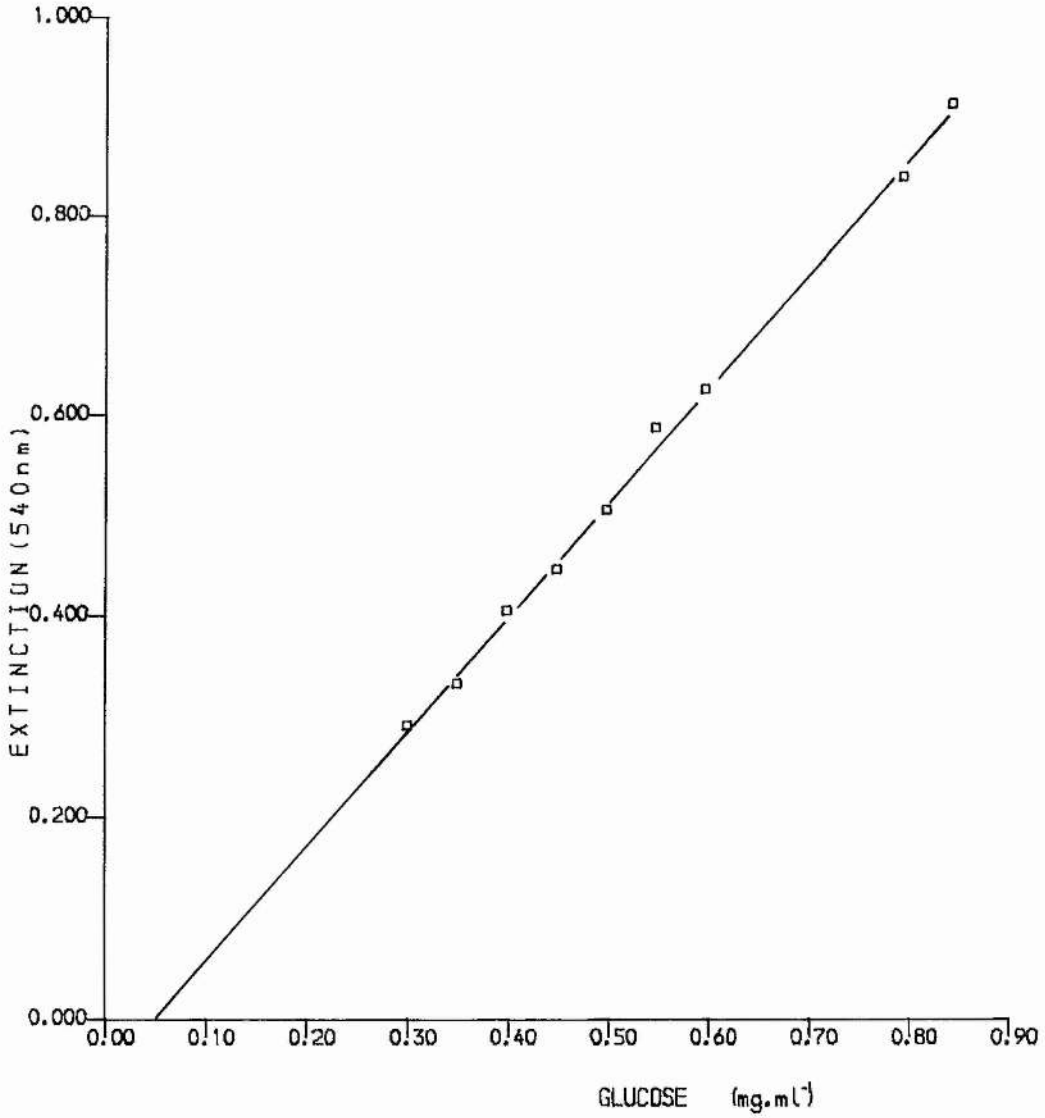


Fig. 3.3.1.2.2. Standard curve for glucose determination using the automated DNS\ method.

3.3.2. - REDUCING METHOD USING NEOCUPROINE.

The use of neocuproine (2,9-dimethyl-1,10-phenanthroline HCl) for the determination of reducing sugars was first described by Brown (56). Neocuproine specifically chelates with Cu^{++} ion produced by the oxidation of reducing sugars developing a coloured complex.

Dygert et al.(57) have replaced the tartrate in Brown's method by glycine, and have expanded the range of precision of the assay from 5-30 µg to 5-125 µg of glucose.

The neocuproine method as recommended by Dygert et al. (57) is as follows:

Reagents:

i) Solution A: Anhydrous sodium carbonate (40 g) was dissolved in about 600 ml of distilled water. 16 g of glycine were added and, when dissolved, 0.450 g of cupric sulphate pentahydrate was dissolved and the volume made up to 1 litre.

ii) Solution B: 0.12 g of neocuproine was dissolved in 100 ml of distilled water and kept in a brown bottle.

Assay:

2 ml of solution A were added to 1-2 ml of solution containing 5-100 µg of glucose or equivalent. 2 ml of solution B were pipetted into the same tube, which was capped, shaken and placed in a vigorously boiling water bath for 8 min. Subsequently, the tube was cooled, the content diluted to 20 ml with distilled water and its absorbance read at 450 nm against distilled water.

For calibration curve see Fig. 3.3.2.1.

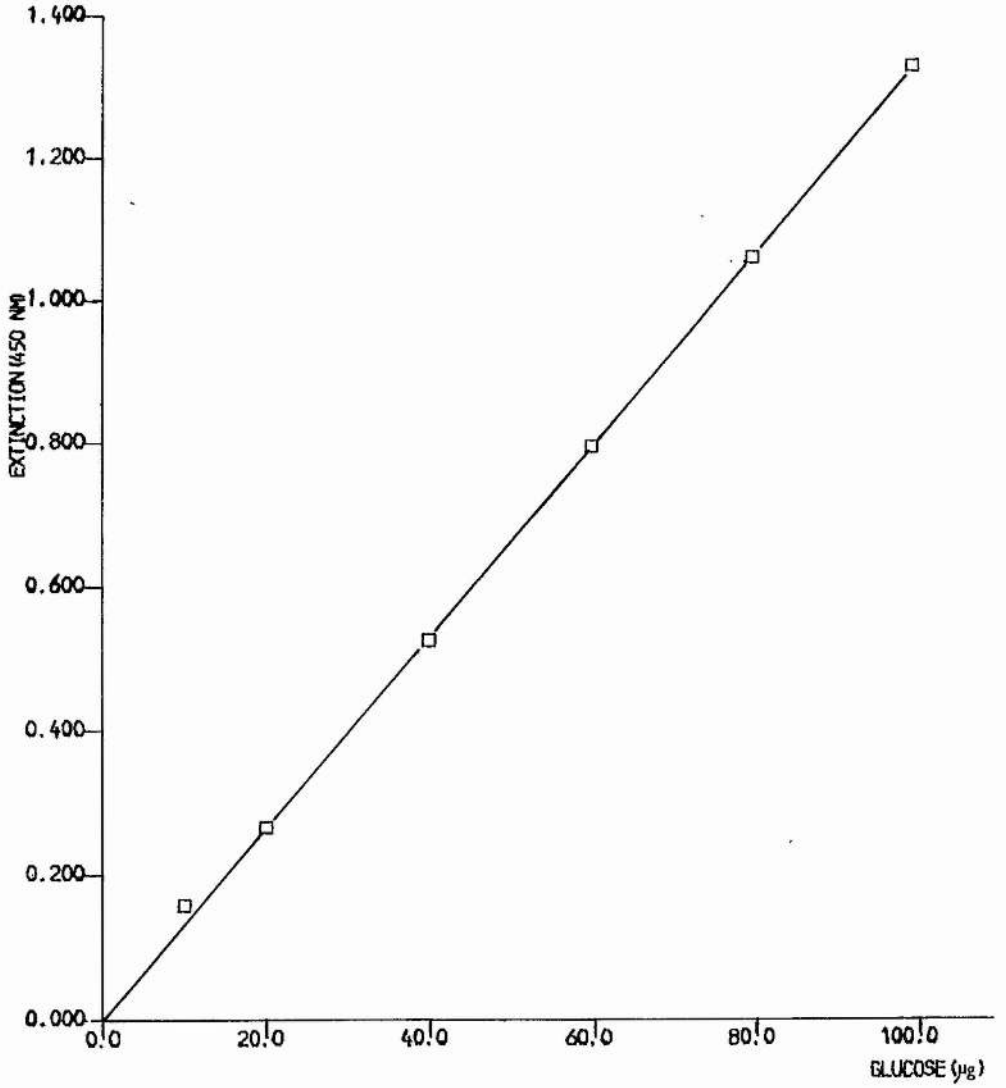


Fig. 3.3.2.1. - Standard curve for glucose determination using the neocuproine method.

3.3.3. - ENZYMIC METHOD OF LLOYD AND WHELAN.

Since Huggett & Nixon (58) published the specific method for the determination of glucose using glucose oxidase-peroxidase system, there has been considerable interest in improving and extending this assay. One of these achievements has been the measurement of glucose in the presence of α-1,4-glucans despite the fact that the impure grades of glucose oxidase contain α-1,4-glucanase activity capable of releasing glucose from such glucans.

The activities of those unwanted enzymes present in glucose oxidase preparations have been overcome by inhibition with Tris (59). A further improvement of this method has been formulated by Lloyd & Whelan (60) replacing Tris-HCl buffer (little buffering capacity below pH 7.0) with Tris-phosphate buffer. This latter assay is as follows:

Reagents:

i) Tris-phosphate-glycerol buffer: Tris (18.15g) and sodium dihydrogen phosphate monohydrate (25g) were dissolved in 150 ml of distilled water. 200 ml of glycerol were added and the pH was adjusted to 7.0 by diasolving the acidic component of the buffer. Finally, the volume was made up to 500 ml with distilled water.

ii) Glucose oxidase-peroxidase reagent: 30 mg of glucose oxidase type II, 3 mg of peroxidase type crude and 10 mg of o-dianisidine dihydrochloride were dissolved in 100 ml of Tris-phosphate-glycerol buffer.

Assay:

To 1 ml of a solution containing not more than 70 ug glucose, 2 ml of glucose oxidase-peroxidase reagent were added. Then, the tube was shaken and incubated at 37° for 30 min. After this time, 4 ml 5 M hydrochloric acid were added, mixed well and the absorbance read at 525 nm.

Fig. 3.3.3.1 shows calibration curve for this assay of glucose.

3.4. - CONTINUOUS ASSAY OF AMYLOGLUCOSIDASE.

3.4.1. - ASCORBIC ACID METHOD.

It is very important in enzyme kinetic studies that the method used for measuring velocities is accurate and preferably rapid, and if initial velocities are measured, it is advantageous to use a continuous recording. Therefore, the need for an accurate, rapid, direct method for amyloglucosidase assay has become apparent.

Most of the amyloglucosidase activity determinations involve the measurement of the product released - glucose.

The glucose oxidase/peroxidase assay of glucose provides the basic idea of a direct spectrophotometric assay for amyloglucosidase.

The method of Huggett & Nixon (58) for the determination of glucose based on the use of glucose oxidase involves the oxidation of o-dianisidine to a coloured product and its spectrophotometric determination at 420 nm. This method, as a basis for continuous assay of glucose-producing enzymes, suffers from the disadvantage that oxidised o-dianisidine (3,3-dimethoxy-4,4-diiminodiphenoquinone) has a low molecular extinction coefficient ($\epsilon = 4 \times 10^3$ litre.mol^{-1}.cm^{-1} in 25 mM sodium phosphate/citrate buffer, pH 5.0 at 420 nm).

In this method for the direct spectrophotometric determination of amyloglucosidase, o-dianisidine has been replaced by ascorbic acid, which has a high molecular extinction coefficient in the ultra-violet region, while its oxidised form (dehydroascorbic acid) has a negligible absorption in this range.

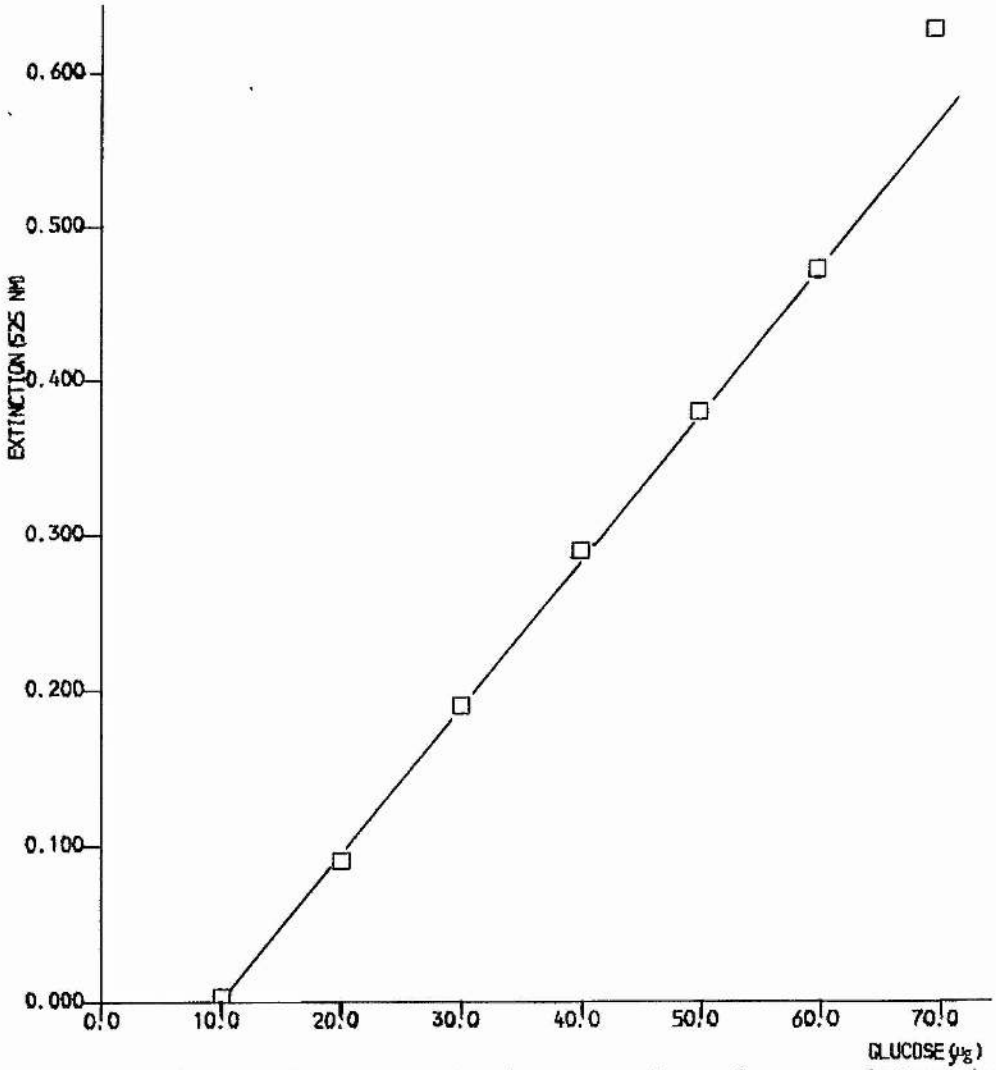


Fig. 3.3.3.1. - Standard curve for glucose determination using the method of Lloyd and Whelan.

This method is based on the following enzyme reactions:

$$\alpha\text{-1,4-glucan} + H_2O \xrightarrow{\text{amyloglucosidase}} \beta\text{-D-glucose} \quad (1)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-}\delta\text{-lactone} \quad (2)$$

$$H_2O_2 + \text{ascorbic acid} \xrightarrow{\text{peroxidase}} H_2O + \text{dehydroascorbic acid} \quad (3)$$

Reagent:

25 mM Phosphate/citrate buffer containing 70 mM oxalate, pH 5.8 (PCO buffer):

98.5 ml of 0.1 M citric acid and 151.5 ml 0.2M dibasic sodium phosphate were mixed and 9.38 g sodium oxalate were dissolved. The pH was then adjusted to 5.8 and the volume made up to 1 litre with distilled water. This buffer has been used successfully to prevent autoxidation of the ascorbic acid (61).

Ascorbic acid reagent: 10 mM L-ascorbic acid was prepared in PCO buffer. The ascorbic acid ultraviolet absorption maximum, which varies with solvent, pH and temperature (62), was 268 nm at pH 5.8 (ϵ =18.4 x 10^3 litre.mol^{-1}.cm^{-1}) at 40° in PCO buffer.

Glucose oxidase-peroxidase reagent: The method of Pazur & Klepp (63) was used to remove α-1,4-glucanase activity from glucose oxidase type II. Purified glucose oxidase and peroxidase type I were dissolved in PCO buffer, to obtain a solution with an activity of about 13 and 1500 units per millilitre, respectively (1 unit = 1 µmol of product released or substrate consumed per minute).

Amylose reagent: 1% (w/v) amylose type I was prepared in PCO buffer.

Assay:

0.5 ml of 0.3 mM ascorbic acid (stock solution diluted 3-times), 1 ml of glucose oxidase-peroxidase reagent and 1.3 ml of amylose reagent were pre-incubated at 40°. 0.2 ml of amylo-

glucosidase containing from 2 to 20 units was introduced into the sample cell and a control cell which lacked ascorbic acid. The decrease in extinction at 265 nm was recorded at a chart speed of 1 cm.min^{-1} for at least 5 min on an SP 800 recording spectrophotometer (Unicam Instruments, Cambridge).

Definition of unit:

The change in extinction per minute may be converted to μmoles glucose released per minute (units) by the equation:

$$\mu\text{moles glucose/min} = (\Delta E/\text{min} \times 3.0)/18.4$$

$\Delta E/\text{min}$: - Change in extinction per minute.

This calculation is based on the use of the molecular extinction coefficient for ascorbic acid of 18.4×10^3 litre.mol^{-1} cm^{-1} and the assumption that 1 mole of ascorbic acid is oxidised per 1 mole of glucose released.

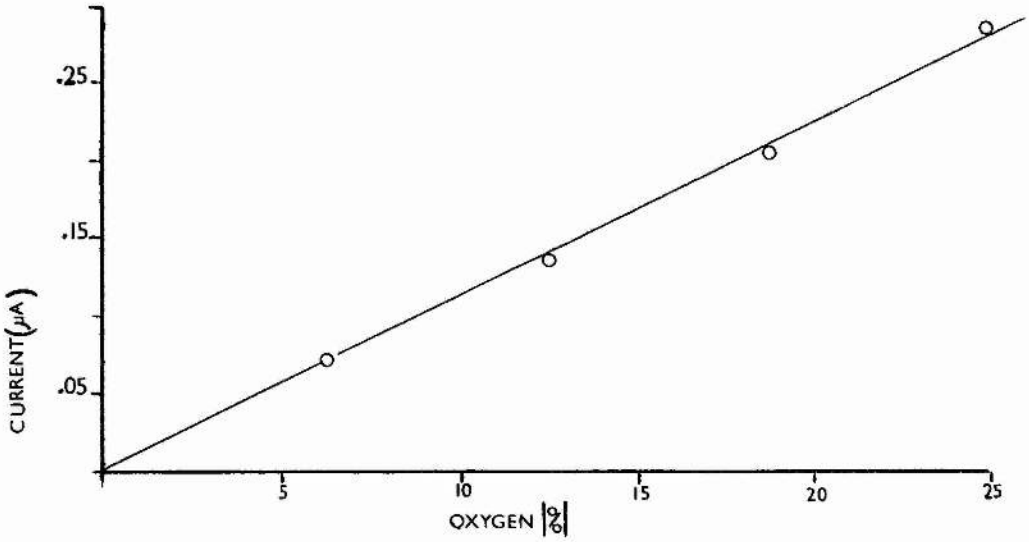
A critical analysis of this method is presented in 4.7.

3.4.2 - GLUCOSE OXIDASE/OXYGEN ELECTRODE METHOD.

In this project, it was used as a Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge) made with a 2 mm diameter cathode, covered with 25 μ thick teflon membrane and filled with saturated KCl solution. The reaction vessel was surrounded by a water jacket through which water from a thermostat was circulated. The current generated was fed into an AR45 recorder 10 mV full scale deflexion (Unicam Instruments, Cambridge).

When the polarising voltage applied to the electrode was varied over the range -0.1 to -1.0 V the electrode showed a "plateau" starting from -0.7 V (See Fig. 3.4.2.1a). At this voltage, there was a linear relationship between the electrode current and the concentration of dissolved oxygen in solution (See Fig. 3.4.2.1b).

The instrument was calibrated by bubbling air into the buffered medium at 40° until saturation was achieved. By using



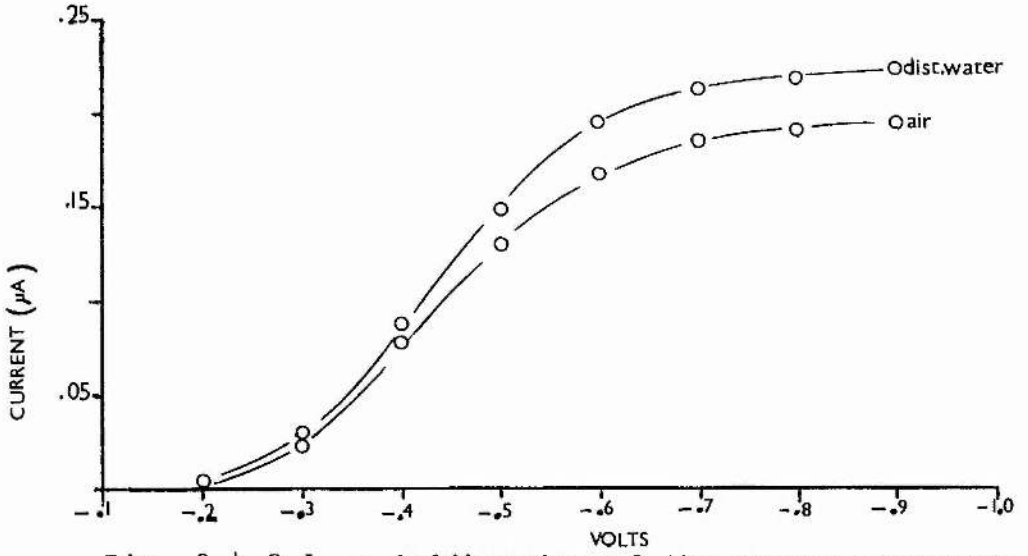


Fig. 3.4.2.1. - Calibration of the oxygen electrode. Relationship between the voltage applied to the oxygen electrode and the current generated.

Fig. 3.4.2.2. - Relationship between the concentration of oxygen and the current generated in the oxygen electrode.

the conversion factor of Chappell (64) - solubility of oxygen of 0.380 μmoles.ml^{-1} - the following calculation was obtained:

$$\frac{\text{volume} \times 0.380\ \mu\text{moles.ml}^{-1}}{88\ \text{scale division}} = 8.636\ \text{nmoles.division of recordor}^{-1}$$

The application of the oxygen electrode for the determination of amyloglucosidase activity is based on the oxygen uptake following the oxidation of the product released (glucose), catalysed by the action of glucose oxidase. These steps are as follows:

$$\alpha\text{-1,4-glucan} + H_2O \xrightarrow{\text{amyloglucosidase}} \beta\text{-D-glucose} \quad (1)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-}\delta\text{-lactone} \quad (2)$$

where the uptake of oxygen in step 2 is followed by oxygen electrode procedure.

Glucose and amyloglucosidase activity were estimated as follows:

Reagent:

i) 25 mM Phosphate/citrate buffer, pH 5.0 (PC buffer): 121.5 ml 0.1M citric acid and 128.5 ml 0.2M dibasic sodium phosphate were mixed, pH adjusted to 5.0 and the volume made up to 1 litre.

ii) Glucose reagent: 0.1% (w/v) glucose was made in PC buffer.

iii) Amylose reagent: 1% (w/v) amylose type I was made in PC buffer.

iv) Glucose oxidase reagent: 0.3 mg.ml^{-1} glucose oxidase grade I was made in PC buffer. This enzyme preparation contains less than 0.01% α-1,4-glucanase and β-fructofuranosidase activities.

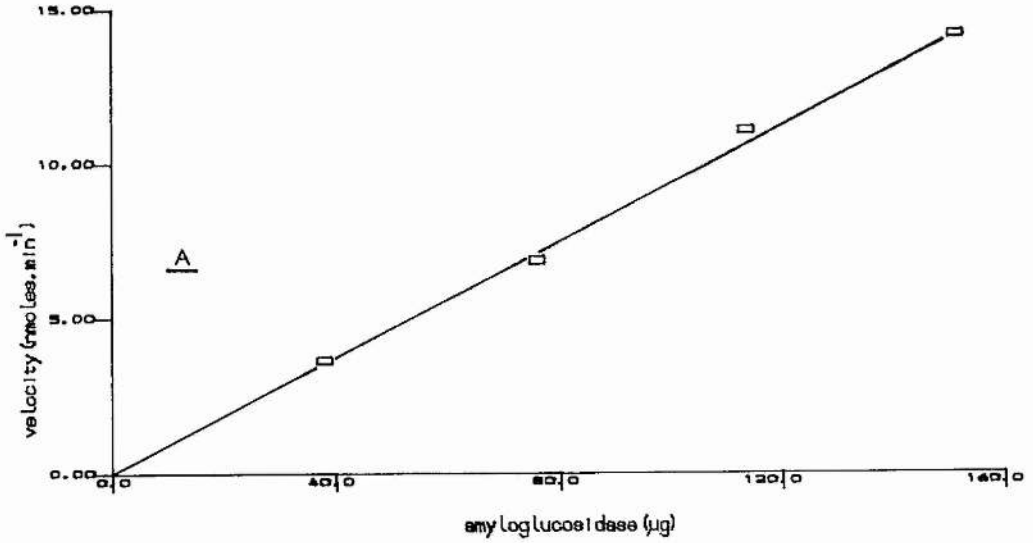
v) Amyloglucosidase reagent: 0.76 mg.ml^{-1} amyloglucosidase (purified as described in 4.1.5) was made in PC buffer.

Glucose assay:

2.0 ml of glucose containing 100-700 µg were incubated in the reaction vessel of the oxygen electrode at 40°. 30 µl of glucose oxidase reagent was added through the hole in the plunger by microsyringe and the oxygen uptake recorded with a chart speed of 1 $min.cm^{-1}$. See Fig. 3.4.2.2b for calibration curve.

Amyloglucosidase assay:

2.0 ml of amylose reagent and 50 µl of glucose oxidase reagent were incubated in the reaction vessel of the oxygen electrode at 40°. 50-200 µl (about 4 to 15 µunits) of amyloglucosidase reagent were added following the same procedure as for glucose oxidase in the glucose assay. Fig. 3.4.2.2a presents the relationship between the initial rate of oxygen uptake and amyloglucosidase concentration.



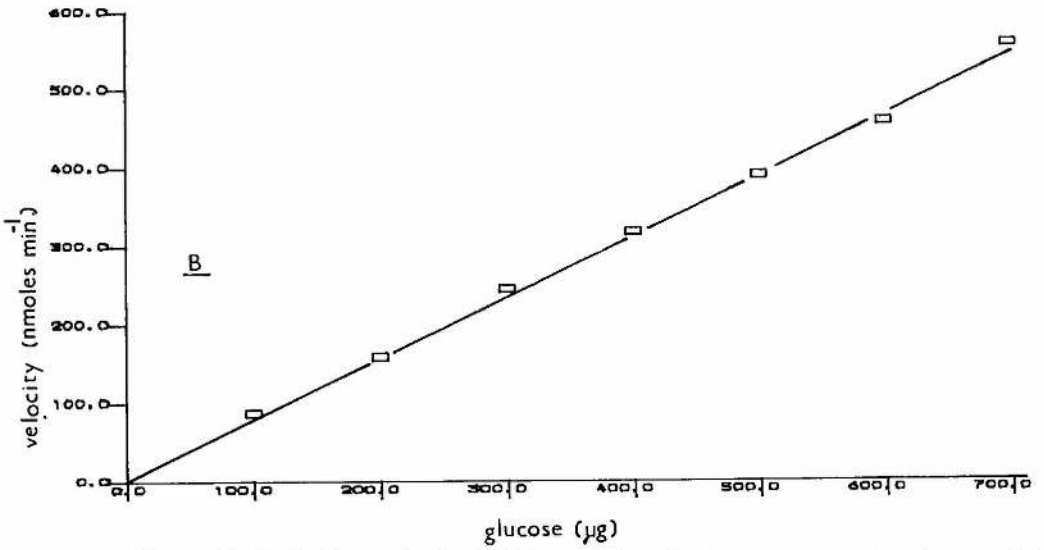


Fig. 3.4.2.2. A) Relationship between amyloglucosidase concentration and the initial rate of oxygen uptake using glucose oxidase/ oxygen electrode method.

B) Standard curve for glucose determination using the glucose oxidase/oxygen electrode method.

3.5. - PROTEIN DETERMINATION.

3.5.1. - Biuret method.

This procedure (65) is as follows:

Biuret reagent:

Cupric sulphate pentahydrate (0.15 g) and sodium potassium tartrate (0.6 g) were dissolved in about 50 ml of distilled water. A solution of 10% (w/v) sodium hydroxide (30 ml) was slowly added with stirring and the volume was made up to 100 ml with distilled water.

Assay:

To 1 ml of a solution containing not more than 10 mg. of protein 4 ml of Biuret reagent was added and after 30 min at 20-25° (room temperature) the absorbance was measured at 540 nm against a blank in which water replaced the protein solution.

A calibration curve was established using casein as a standard protein (See Fig. 3.5.1.1.).

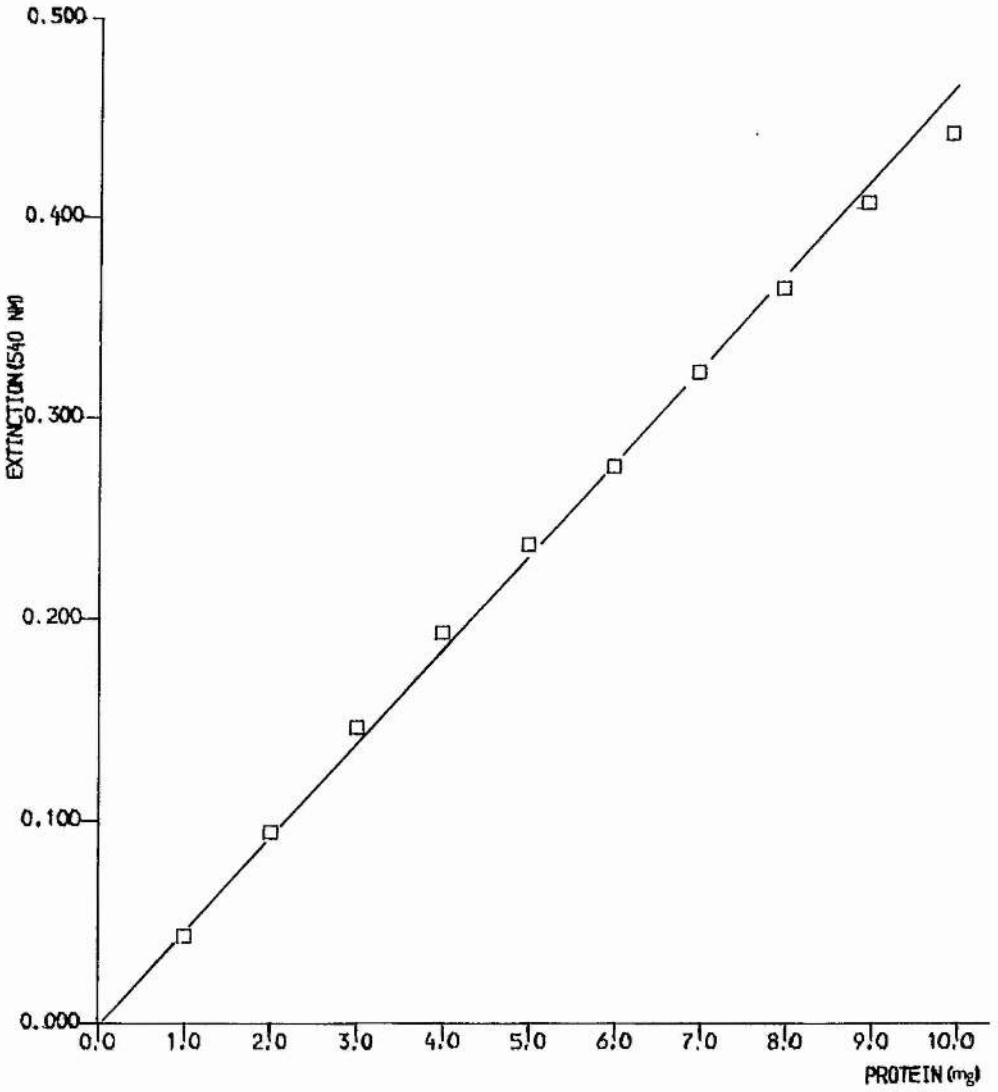


Fig. 3.5.1.1. - Standard curve for the determination of protein by the Biuret method.

3.5.2. -METHOD OF LOWRY ET AL.

Protein measurement with Folin Ciocalteu's phenol reagent according to Lowry et al. (66) involves two steps: reaction with copper in alkaline solution and reduction of Folin reagent. This method is as follows:

Reagent:

A reagent: Sodium carbonate (10 g) and sodium hydroxide (50 ml 1M) were dissolved in about 300 ml of distilled water and the volume made up to 500 ml with distilled water.

B reagent: Cupric sulphate pentahydrate (0.5 g) and sodium potassium tartrate (0.2 g) were dissolved in 90 ml of distilled water and sodium hydroxide (10 ml 1M) was added.

C reagent: 50 ml A reagent and 1 ml B reagent were mixed. This reagent was discarded after 24 h.

D reagent: Folin Ciocalteu's phenol reagent 1.87 N was made up to 1N with distilled water.

Procedure:

2 ml of C reagent were added to 5-60 µg of protein contained in a volume up to 2.8 ml and allowed to stand for 10 min or longer at room temperature. 0.2 ml of D reagent was added, immediately mixed, and the volume made up to 5 ml with distilled water. After 30 min or longer the absorbance was measured at 750 nm (5-20 µg of protein) or 500 nm (greater than 20 µg of protein).

Fig. 3.5.2.1. shows standard curves using lysozyme grade I (3x cryst.) as a standard protein.

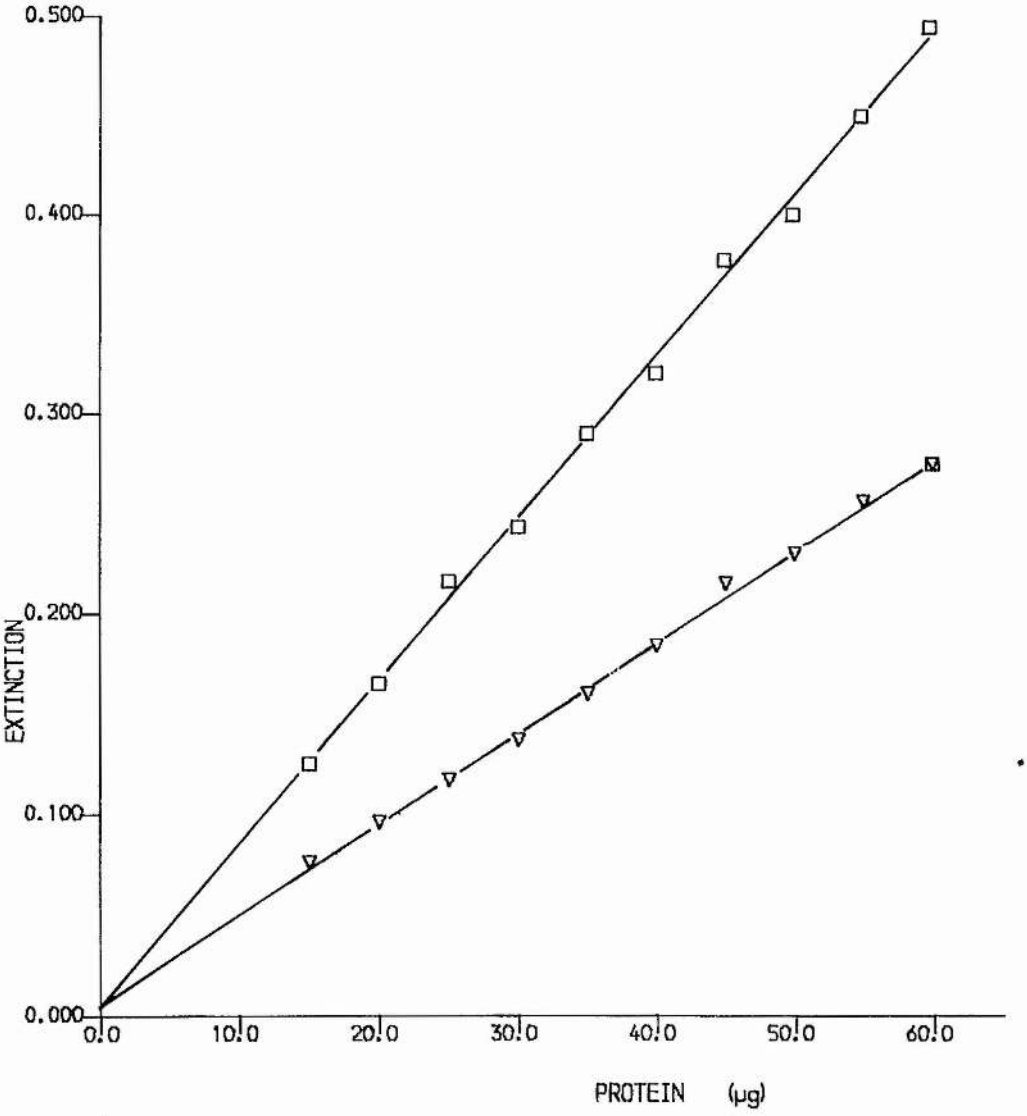


Fig. 3.5.2.1. Standard curve for the determination of protein by the method of Lowry et al. Extinction was read at 750 nm (□) and 500 nm (▽).

3.5.3.- METHOD OF WARBURG & CHRISTIAN.

Protein estimations based on ultraviolet absorption measurements were carried out according to recommendations of Warburg & Christian (67). Absorbance measurements were made at 280 nm and 260 nm and calculations were according to a modified Kalckar's equation (68) as follows:

$$\text{Protein } (mg.ml^{-1}) = 1.55 \times E_{280} - 0.76 \times E_{260}$$

3.6. - DETECTION OF α-AMYLASE IN AMYLOGLUCOSIDASE PREPARATIONS.

3.6.1. - PARTIALLY OXIDISED AMYLOSE METHOD.

The detection of α-amylase activity in amyloglucosidase preparations according to the method described by Marshall & Whelan (32) was performed as follows:

5% oxidised amylose (2.5 mg), an appropriate amount of enzyme and 50 mM acetate buffer, pH 5.0 (1.5 ml), were incubated at 40° together with a reference digest containing unoxidised amylose. Aliquots (50 μl) were removed at appropriate time intervals for glucose determination by Lloyd and Whelan's method.

As an example, β-amylase type II-B (9.2 units) from barley free of α-amylase was employed and the extent of hydrolysis of both oxidised and unoxidised amylose by this enzyme preparation can be seen in Fig. 3.6.1.1.

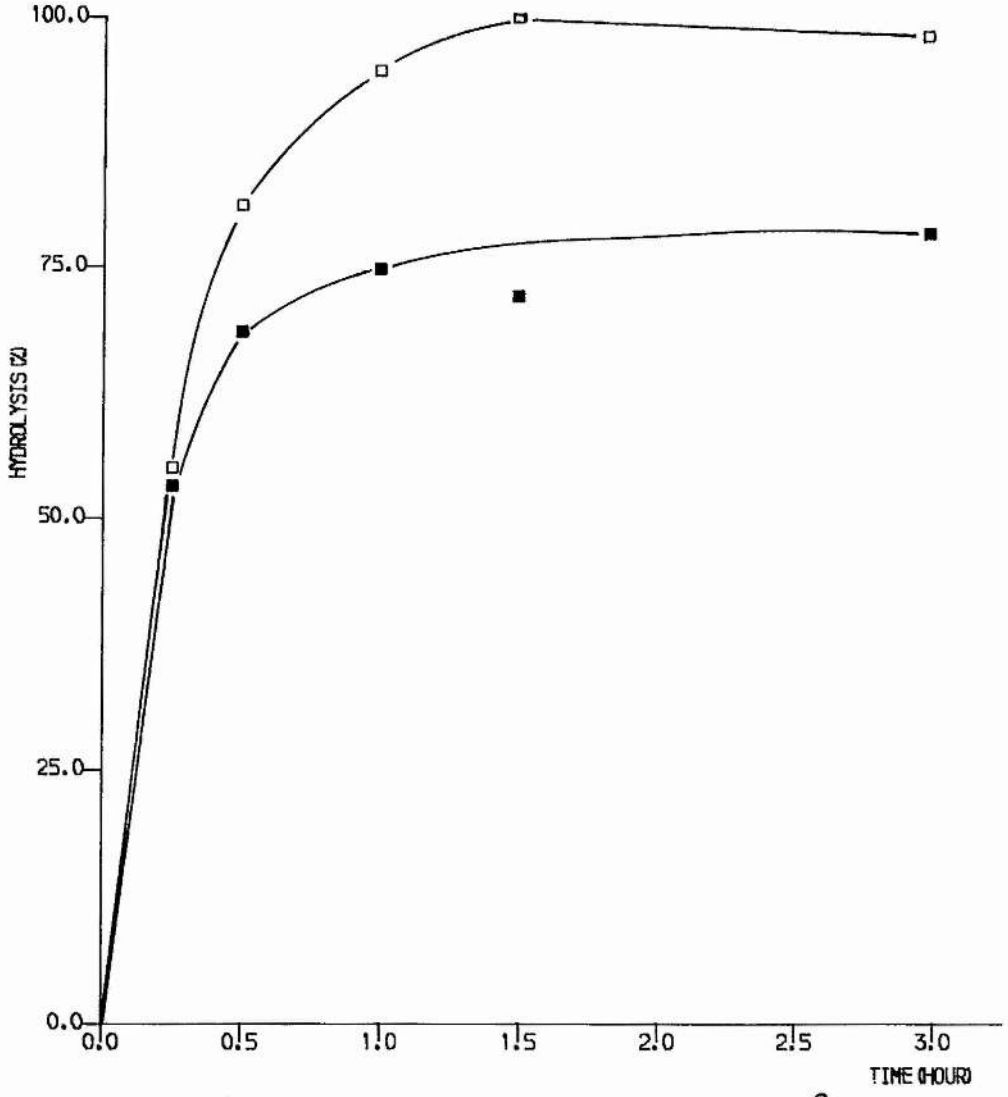


Fig. 3.6.1.1. Time course of action of β -amylase from barley on amylose (□) and 5^{c} oxidised amylose (■).

3.6.2. - CHROMOGENIC SUBSTRATE METHOD.

The determination of coloured products released from chromogenic substrates by enzyme catalysis was carried out as follows:

Amylose azure reagent:

0.2% (w/v) amylose azure B grade was suspended in 25 mM phosphate/citrate buffer, pH 5.8 containing 0.1% (w/v) bovine serum albumin and heated in a boiling water bath for 5 min to give a colloidal suspension, providing therefore a larger substrate surface area. The inclusion of albumin is to prevent spontaneous hydrolysis.

Assay:

4.9 ml of amylose azure reagent and 0.1 ml of the enzyme preparation were incubated at 40° under stirring, and aliquots of 1.0 ml were withdrawn at time intervals and centrifuged at 600 g for 5 min. The absorbance of the supernatants was measured at 595 nm against a blank in which water replaced the enzyme.

100% hydrolysis of 1.0 ml of 0.2% (w/v) amylose azure gives an extinction of 0.4 at 595 nm and releases 620 µg of glucose.

Amylopectin azure reagent and assay:

0.2% (w/v) amylopectin azure A grade was prepared in the same way as amylose azure reagent and the assay procedure was identical to that described above for amylose azure.

100% hydrolysis of 1.0 ml of 0.2% (w/v) amylopectin azure gives an extinction of 0.3 at 595 nm and releases 300 µg of glucose.

3.7. - PERIODATE OXIDATION OF AMYLOSE.

3.7.1. - PARTIAL OXIDATION OF AMYLOSE.

Sodium metaperiodate was used to oxidise amylose to obtain 5 different degrees of oxidation. These oxidised polysaccharides were obtained as follows:

Amylose type I (500 mg) was dissolved in 40 ml of distilled water, and a solution (10 ml) containing the calculated amount of sodium metaperiodate necessary to give the various degrees of oxidation was slowly added with stirring. The mixture was kept for 4 h in darkness, dialysed against water overnight and freeze-dried.

By using the following equation:

$$\text{mg } NaIO_4 = \frac{\text{mg amylose} \times \% \times 214}{16{,}200}$$

% = % degree of oxidation sought.

Values of 33, 99, 198, 297 and 396 mg of sodium metaperiodate were estimated to give 5, 15, 30, 45 and 50% oxidation of amylose, respectively.

To determine the actual extent of oxidation obtained, 10 mg of each oxidised amylose were dissolved in 4.0 ml 1 M HCl and incubated at 100° for 3 h. 4.0 ml 1 M NaOH were then added, pH adjusted to 7.0 and the volume was made up to 10 ml with distilled water.

Finally, the glucose content of these hydrolysates were determined by the method of Lloyd and Whelan. A control containing 1.1 mg of glucose was processed in the same way to estimate the loss of glucose during the acid hydrolysis. Table 3.7.1.1 shows the amount of glucose recovered after the acid hydrolysis of each oxidised amylose.

TABLE 3.7.1.1. - AMOUNTS OF GLUCOSE OBTAINED FROM ACID HYDROLYSIS OF UNOXIDISED AND OXIDISED AMYLOSES AS DETERMINED BY THE ENZYMIC METHOD RECOMMENDED BY LLOYD AND WHELAN.

Theoretical oxidation (%)	Glucose in the samples* (mg/ml)	Glucose in the hydrolysates @ (mg/ml)	Recovered glucose (%)	Oxidised glucose (%)
0	1.11	1.01	100.0	0
5	1.11	0.96	95.4	4.6
15	1.11	0.83	82.1	17.9
30	1.11	0.70	69.3	30.7
45	1.11	0.54	53.4	46.6
60	1.11	0.35	34.8	65.2
glucose	1.11	1.00	&	

* - Calculated by the formula

$$\text{mg glucose/ml} = \text{mg amylose} \times \frac{180}{162}$$

@ - The glucose content in the hydrolysates was estimated by Lloyd and Whelan's method.

& - 10% of glucose is lost during the acid hydrolysis.

3.7.2. - TOTAL OXIDATION OF AMYLOSE-DETERMINATION OF DEGREE OF POLYMERISATION (DP)

The degree of polymerisation of amylose was estimated by determining the non-reducing end groups of the molecule by the periodate oxidation method.

This method is based on the release of two moles of formic acid per reducing end and one mole of formic acid per non-reducing end during the oxidation of amylose by the periodate ions Therefore, the number of glucose monomers per chain can be calculated, since the number of end groups is stochiometrically related to the moles of formic acid produced. Obviously, if an ensemble of polymers of various degree of polymerisation is used, the result will be the number-average degree of polymerisation ($\bar{DP}$) of the sample.

This method is as follows:

800 mg of amylose were dissolved in 100 ml of distilled water and cooled at 4^{o}. 20 ml of 0.37M $NaIO_4$ were added. This mixture was divided into 2 aliquots.

To one aliquot of 60 ml, 1 ml of ethylene glycol was added with stirring and the pH measured and noted after 30 min in darkness.

The second aliquot of 60 ml was stored at 4^{o} in darkness for 24 h, after which 1 ml of ethylene glycol was added with stirring, and after 30 min in the dark the pH was measured again. The mixture was then titrated back to the original pH of aliquot I with 0.001M NaOH. The release of formic acid and the $\bar{DP}$ were calculated as below:

moles formic acid released = vol. x 10^{-6} moles.

moles glucose = wt. amylose/162 moles.

chain number = moles formic acid released/3.

$\bar{DP}$ = moles glucose/chain number,

where

vol.: = volume of 0.001M NaOH.

wt. amylose: = weight of amylose employed.

For amylose type I from potato a volume of 9.3 ml of 0.001M NaOH was used to titrate aliquot 2. Thus $\overline{DP}$ equal to 797 was found according to the following calculations:

moles formic acid released = 9.3×10^{-6} moles.

moles glucose = $0.4/162 = 2.47 \times 10^{-3}$ moles.

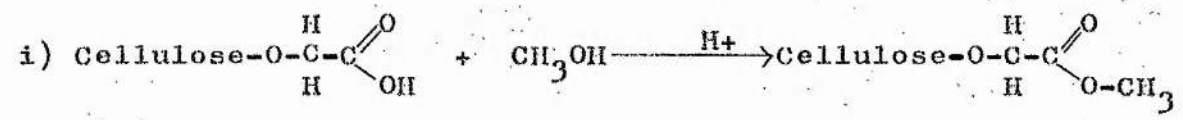
chain number = $9.3 \times 10^{-6}/3 = 3.1 \times 10^{-6}$ chains.

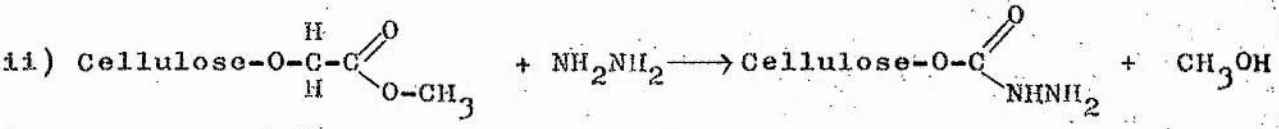
$\overline{DP} = 2.47 \times 10^{-3}/3.1 \times 10^{-6} = 797$ moles glucose/chain.

3.8. - METHODS OF IMMOBILISING AMYLOGLUCOSIDASE.

3.8.1. - PREPARATION OF CARBOXYMETHYLCELLULOSE HYDRAZIDE.

The preparation of CM-cellulose hydrazide from CM-cellulose was carried out by the procedure recommended by Crook et al. (69). This method is based upon the two following reactions:





i) Preparation of CM-cellulose methyl ester:

20 g of CM-cellulose CM-11 (0.6 mequiv.g^{-1}) and 300 ml of dry methanol were stirred together at 70°, 2 ml 10 M HCl were then added and this mixture was refluxed with stirring for 4 h.

After 4 h. the resultant CM-cellulose methyl ester was washed three times with 100 ml of dry methanol and dried on a vacuum filter.

ii) Conversion of the methyl ester group into hydrazide:

The washed powder from the above reaction and 200 ml of dry methanol were stirred together at 40°, 20 ml of hydrazine hydrate were then added and the mixture was incubated at 40° for 72 h.

The product was twice washed with 100 ml of dry methanol, methanol saturated with CO_2, 90% aqueous methanol and dry methanol, successively.

The IR-spectra of the CM-cellulose hydrazide so obtained was compared with a sample purchased from Sigma Chem. Co. London (See Fig. 3.8.1.2.)

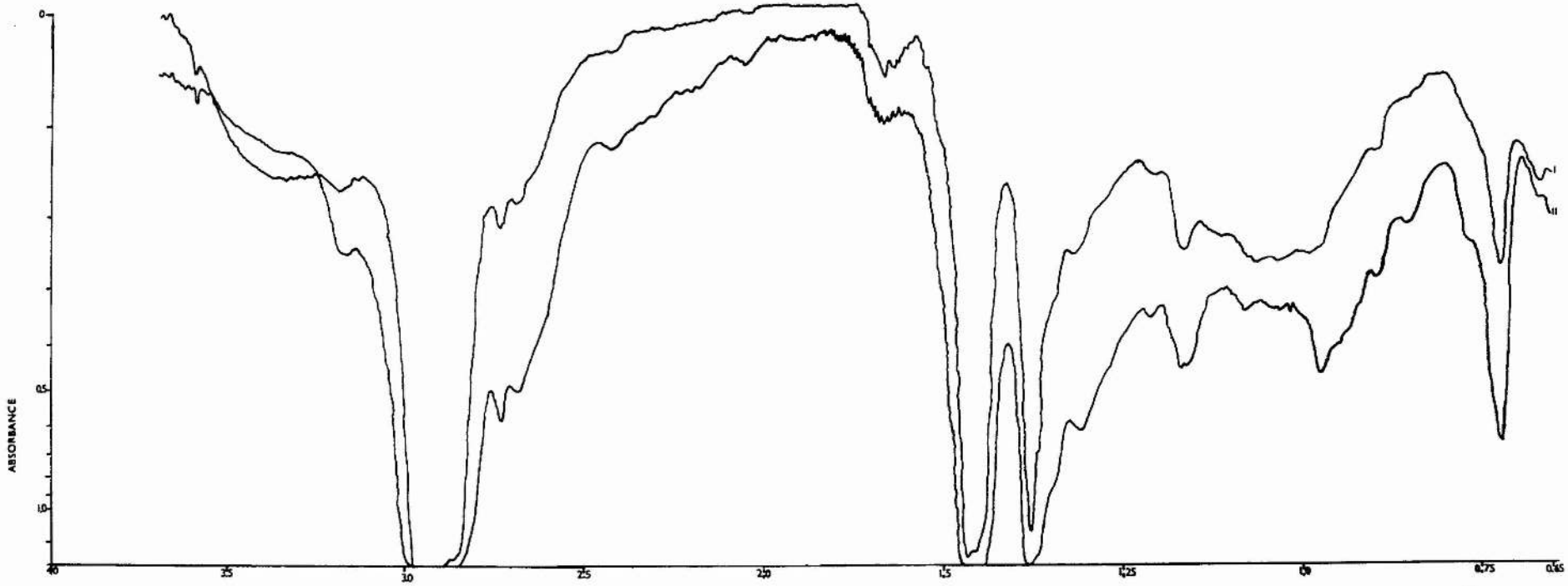


Fig.3.8.1.2. - Comparison between the IR-spectra of a commercial CM-cellulose(I) and that prepared as in 3.8.1.(II).

3.8.2. - IMMOBILISATION OF AMYLOGLUCOSIDASE ON CARBOXYMETHYL-CELLULOSE HYDRAZIDE.

An immobilised amyloglucosidase on CM-cellulose hydrazide was prepared according to the method of Christison (44). This method involves reaction between carbonyl (aldehyde) residues formed after periodate oxidation of the carbohydrate prosthetic group of amyloglucosidase and carbonyl-reactive groups (hydrazide) of the CM-cellulose derivative.

i) Oxidation of the carbohydrate prothetic group of amyloglucosidase:

120 mg of amyloglucosidase were dissolved in 4.0 ml 50 mM sodium acetate buffer, pH 4.8. 3.2 ml 60 mM sodium metaperiodate were then added with stirring and the mixture was kept in darkness for 1.5 h at 23°. After this time, 2 ml of 3% (w/v) glucose solution were added to inactivate the remaining periodate and the solution was dialysed against 10 mM sodium acetate buffer, pH 4.8 overnight at 4°.

Assuming the mol.wt. of amyloglucosidase is 100,000 (9), 120 mg contains 1.2 μmoles of enzyme. Thus 96 μmoles of carbohydrate residues are available for oxidation since there are 80 μmoles of carbohydrate residues per mole of enzyme according to Pazur et al. (18). Therefore, to provide the ratio periodate to carbohydrate 2:1, as recommended by Christison (44), 192 umoles of periodate ions (3.2 ml of 60 mM $NaIO_4$) are necessary.

ii) Immobilisation of oxidised amyloglucosidase on CM-cellulose hydrazide:

The pH of the dialysed oxidised amyloglucosidase preparation was adjusted to 6.5 and 600 mg of CM-cellulose hydrazide were added and the suspension was stirred for 48 h at 4°.

The CM-cellulose oxidised amyloglucosidase immobilised derivative was washed with 1.0M NaCl, 0.5M $NaHCO_3$ and 50 mM

phosphate/citrate buffer, successively, to remove proteins physically attached to the support. This washing was different from that used by Christison (50 mM phosphate/citrate buffer containing 0.5M sodium chloride).

3.8.3. - IMMOBILISATION OF AMYLOGLUCOSIDASE ON p-AMINO-BENZYLCELLULOSE.

PAB-cellulose was pretreated (diazotised) as follows:

500 mg of PAB-cellulose (0.11 mequiv.NH_2/g) were stirred in 100 ml of 0.6N HCl at 4^o and 0.2 g of $NaNO_2$ were added slowly to the suspension. After 20 min. the powder was filtered and washed with distilled water until pH was nearly neutral.

Immediately after, this diazotised cellulose derivative was introduced into 10 ml of amyloglucosidase (containing 2 mg/ml) in borate buffer, pH 8.4 at 4^o and stirred overnight. The enzyme physically coupled to the PAB-cellulose was then removed by washing the powder with 1.0 M NaCl, 0.5 M $NaHCO_3$ and 25 mM phosphate/citrate buffer, successively.

Finally, the resultant material (amyloglucosidase covalently attached to PAB-cellulose) was resuspended in 25 mM phosphate/citrate buffer, pH 5.8 and stored at 4^o.

3.8.4. - DETERMINATION OF THE ACTIVITY OF IMMOBILISED AMYLO-GLUCOSIDASE.

The activity of amyloglucosidase attached to either CM-cellulose or PAB-cellulose was determined as follows:

The insoluble material, containing the enzyme covalently bound to it, was resuspended in the required concentration of substrate; volume of solution; ionic strength and pH given by an appropriate buffer and stirred at a constant rate at 40°. Samples were removed at time intervals and centrifuged at 600 x g. The glucose content of the supernatant was determined by the glucose oxidase/oxygen electrode method, whereas the precipitates were collected together and washed with 1M sodium chloride and the used buffer, successively. Then, the recovered precipitates were used again.

3.9. - SUPPLEMENTARY METHODS.

3.9.1. - GLUCOSE OXIDASE PURIFICATION.

A glucose oxidase purification recommended by Pazur & Kleppe (63) was carried out as follows:

Preparation of chromatographic columns:

DEAE-cellulose DE-32 (40 g) was stirred into 1 litre 0.5M HCl and left for 30 min. After this first treatment, it was washed in a funnel fitted to a Buchner flask in which vacuum was applied, until the effluent was at pH 4.0. Then the ion exchanger was resuspended in 1 litre 0.5M NaOH and again left for 30 min. The result from this second treatment was also washed in a funnel/Buchner flask/vacuum system until the filtered effluent was about pH 7.0.

This pre-cycled DEAE-cellulose and about 200 ml 0.2M acetic acid were stirred together with a magnetic stirrer in a stoppered Buchner flask and vacuum was set.

The pre-cycled and degassed DEAE-cellulose was dispersed in 1.2 l 50 mM sodium acetate buffer, pH 4.5 and allowed to settle in a measuring cylinder for 1 h. After that, the supernatant buffer solution containing fines was removed. The DEAE-cellulose so obtained was divided into 2 parts: 75% of this ion exchanger was introduced into a column (400 x 35 mm) and 25% into a smaller column (200 x 20 mm). Both columns were washed with 50 mM sodium acetate buffer, pH 4.5 until equilibration.

Sample application:

500 mg of glucose oxidase type II were dissolved in 5 ml of distilled water and dialysed against 10 mM sodium acetate buffer, pH 4.5, overnight at 4°. This dialysed enzyme sample was made up to 10 ml with the same buffer and applied to the top of the first column (400 x 35 mm).

Elution procedure:

The column was washed with 0.5l 70 mM sodium acetate buffer, pH 4.5 and subsequently with 0.5l 100 mM sodium acetate

buffer, pH 3.7 at a flow rate of 90 ml/h. The effluent was monitored at 258 nm with a Uvicord Spectrophotometer (LKB Inst., Sweden). The protein content of the peaks collected was determined by the method of Lowry et al. (66), whereas the enzymatic activities were assayed as follows:

∝-1,4-glucanase activity in the glucose oxidase preparation:

in an assay mixture composed of:

0.3 mM L-ascorbic acid (0.5 ml) prepared to PCO buffer.

1% (w/v) amylose (1.5 ml) prepared in PCO buffer.

150 µg/ml peroxidase type I (0.9 ml) prepared in PCO buffer,

0.1 ml of the peak was added and the absorbance at 268 nm was recorded continuously at a chart speed of 1 cm/min against a reference lacking ascorbic acid. The reaction was performed at 40° in cells of 1 cm light-path in a SP 800 spectrophotometer (Unicam Instruments, Cambridge). This procedure, based on the ascorbic acid method (See 3.4.1.), would measure ∝-1,4-glucanase activity in the peak containing glucose oxidase activity.

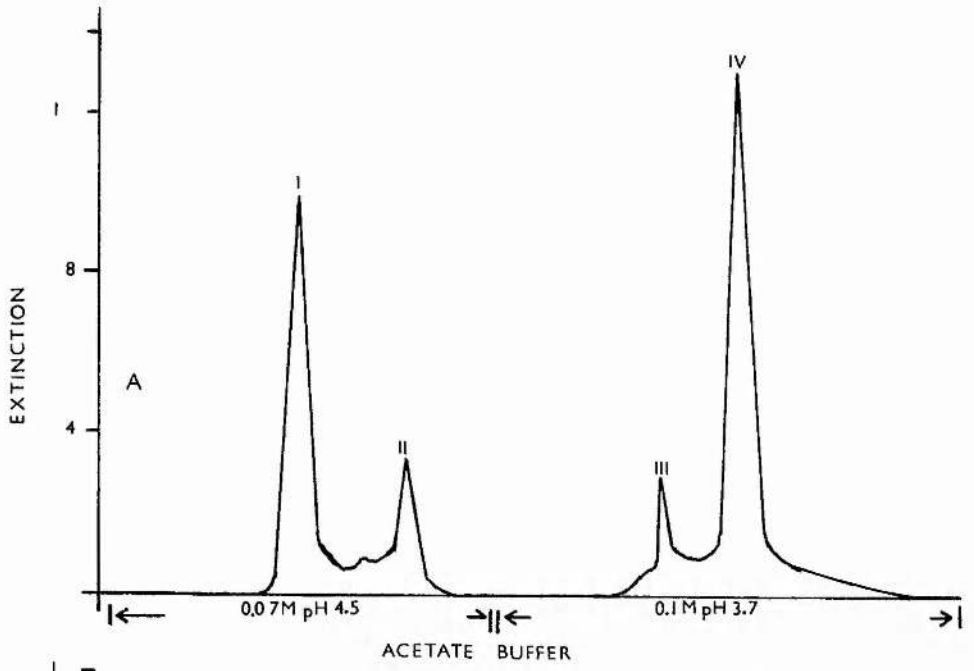
Glucose oxidase activity:

Glucose oxidase activity was properly estimated by adding 0.1 ml of 0.2% (w/v) glucose solution to the reaction cell and recording the reaction at 268 nm as above.

Catalase activity:

Catalase determination was processed by the modified Chance's method (See 3.9.2.).

A representative elution profile of this fractionation is shown in Fig. 3.9.1.1.a. The peak containing the bulk of glucose oxidase activity (4th peak) was applied on the top of the second column (200 x 20 mm) and rechromatographed by the same procedure as for the first column. The pattern of this second chromatography is presented in Fig. 3.9.1.1.b.



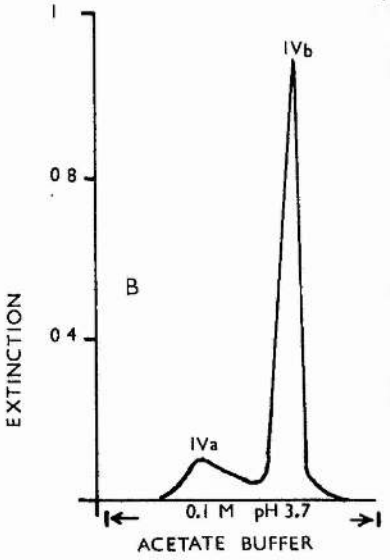


Fig. 3.9.1.1. A) Elution profile of the chromatography of glucose oxidase type II on a DEAE-cellulose column(400 x 35 mm). Stepwise elution was performed as indicated in the chromatogram and recommended by Pazur & Kepple(63). The eluate was examined for protein by spectrophotometry at 258 nm. Glucose oxidase, ∝-1,4-glucanase and catalase activities were determined in the peaks obtained and the results are presented in Table 3.9.1.1.

B) Elution profile of the chromatography of the 4th peak from the above chromatography on a second DEAE-cellulose column(200 x 20 mm). Stepwise elution was performed as indicated in the chromatogram(63) and the eluate was analysed as above.

A summary of this purification procedure is displayed in Table 3.9.1.1.

Table 3.9.1.1. - ELUTION OF GLUCOSE OXIDASE, α-1,4-GLUCANASE AND CATALASE FROM GLUCOSE OXIDASE TYPE II PREPARATION ACCORDING TO THE METHOD OF PAZUR & KLEPPE (63).

peak*	Specific activities (units.mg protein^{-1})		
	Glucose Oxidase	α-1,4-glucanase	catalase
1	0.040	0.333	6.500
11	0.0	0.0	4.728
111	0.200	0.100	3.268
1V	0.368	0.034	1.927
1Va	0.223	0.166	1.927
1Vb	1.283	0.0	3.509

* - Obtained from chromatography on DEAE-cellulose (See Fig. 3.9.1.1.).

3.9.2. - CATALASE ASSAY.

Catalase assay was performed by a slight modification of the method of Chance (69), in which the decrease in extinction at 240 nm is measured when catalase and H_2O_2 are incubated together. This assay is as follows:

To a 3 ml cuvette were added 3 ml of 88 mM H_2O_2 in 10 mM Tris-chloride buffer, pH 7.0. 0.01 ml of the peak was then pipetted onto the side wall of the cuvette which was covered with parafilm, mixed by inversion, and inserted into the SP 800 spectrophotometer (Unicam Instruments, Cambridge). Recording against a reference cuvette of 10 mM Tris-chloride buffer, pH 7.0 was started immediately at a chart speed of 1 cm/10 s.

4. - RESULTS AND DISCUSSION.

4.1. - STUDIES ON THE PURIFICATION OF AMYLOGLUCOSIDASE.

4.1.1. - AMMONIUM SULPHATE FRACTIONATION.

A summary of the data for a typical ammonium sulphate fractionation of amyloglucosidase is given in the table 4.1.1.1. The bulk (80%) of the enzyme was recovered in the step containing 60-70% ammonium sulphate.

4.1.2. - ETHANOLIC PRECIPITATION.

Ethanolic precipitation of protein was used to concentrate amyloglucosidase preparations, as well as to purify them from ethanol soluble contaminants.

A strength of approximately 80% (v/v) ethanol was employed, based on the results shown in Table 4.1.2.1. These percentages of ethanol were obtained as follows:

A :- 2g freeze-dried crude enzyme preparation from Rhizopus delemar fermentation were dissolved in 15ml of distilled water.

B :- 99% (v/v) ethanol.

%	A	B
0 - 79.2	5ml	20ml
0 - 82.5	6ml	30ml
0 - 90.0	2ml	20ml
0 - 94.3	1ml	20ml

After mixing, the proteins were centrifuged at 10,000 x g for 10 min and the precipitates redissolved to their original volumes, namely, the volumes showed in A. Levels for ethanolic percentage higher than 79.2% (v/v) precipitated inactive proteins, thus decreasing the specific activity of the material obtained.

TABLE 4.1.1.1. - PURIFICATION OF AMYLOGLUCOSIDASE FROM RHIZOPUS DELEMAR BY AMMONIUM SULPHATE FRACTIONATION.

Step	Volume of solution (ml)	Protein* (mg/ml)	Total © activity (units)	Specific © activity (units/mgx10^{-3})	Recovery (%)
Crude enzyme	10	41.4	3.06	7.4	100
Ammonium sulphate fractionation					
0 - 20%	1	101.6	0.23	2.3	7.5
20 - 30%	1	29.7	0.23	7.7	7.5
30 - 40%	1	70.0	0.15	2.1	5.1
40 - 50%	1	25.0	0.0	0.0	0.0
50 - 60%	1	30.0	0.15	5.0	5.1
60 - 70%	1	42.0	2.42	57.6	79.0
70 - 100%	10	55.0	0.0	0.0	0.0

* - The protein content of the material obtained was estimated by the procedure of biuret.

© - The enzymic activity was assayed by the DNSA method.

TABLE 4.1.2.1. - ETHANOLIC PRECIPITATION OF AMYLOGLUCOSIDASE FROM RHIZOPUS DELEMAR.

Step	Volume of solution (ml)	Protein* (mg/ml)	Activity@ (units/ml)	Specific activity (units/mg protein)
Crude enzyme&	15	10	0.980	0.098
Ethanol strength				
0 - 79.2%	5	4.0	0.737	0.184
0 - 82.5%	6	4.8	0.708	0.147
0 - 90.0%	2	6.3	0.872	0.138
0 - 94.3%	1	6.0	0.698	0.116

* - The protein content of the material obtained was estimated by the procedure of Lowry et al.

@ - The enzymatic activity was assayed by the glucose oxidase/oxygen electrode method.

& - The crude enzyme was obtained by dissolving 2g of the freeze-dried crude enzyme from Rhizopus delemar fermentation in 15 ml of distilled water.

4.1.3. - CHROMATOGRAPHY ON DIETHYLAMINOETHYLCELLULOSE.

4.1.3.1. - GRADIENT ELUTION.

A method recommended by Smyley et al. (71) was employed.

800 mg of the 60-70% ammonium sulphate fraction were applied to the top of a 350 x 25 mm column of DEAE-cellulose that had been washed with 0.5M HCl, distilled water, 0.5M NaOH, distilled water until the pH value of the washing was below 8.0 and finally 0.1M sodium acetate buffer, pH 4.2 until equilibration. Elution was started with the same buffer and when the first peak was detected a linear gradient elution was set up 0.3 to 1.0M sodium acetate buffer, pH 4.2. The flow rate was 60ml/h. Amyloglucosidase emerged as a single peak and was completely eluted when the gradient was approx. 0.5M. The protein peak was recorded by continuous ultraviolet monitoring of the effluent stream with an LKB-Uvicord. Fractions of 2.5ml were collected and then enzymatic activity determined by the DNSA method.

The tubes containing the bulk of the activity were pooled, dialysed against distilled water overnight at 4^{o} and then concentrated in vacuo (approx. 15 mmHg) at 40^{o}. A typical elution profile can be seen in Fig. 4.1.3.1.1.

In this particular example, a preparation (tubes no. 35-55) containing a specific activity of 0.3 units.mg^{-1} was obtained, whereas the sample applied on the top of the column showed a value of 0.075 units.mg^{-1}. In total, a 40-fold purification was achieved by using this chromatographic step on DEAE-cellulose after ammonium sulphate fractionation.

However, the amyloglucosidase obtained by these methods has been demonstrated to contain traces of α-amylase according to the partially oxidised amylose procedure.

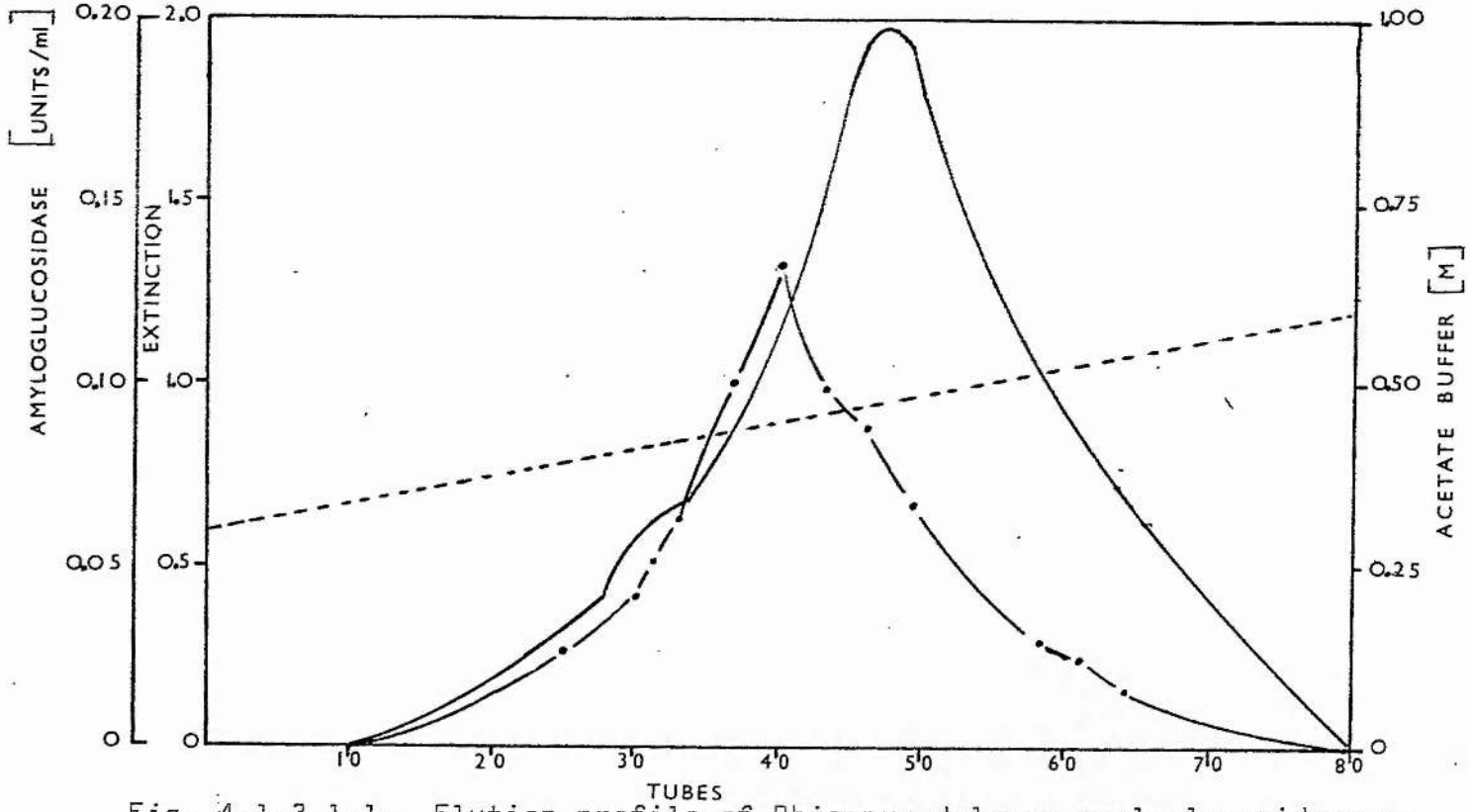


Fig. 4.1.3.1.1. Elution profile of Rhizopus delemar amyloglucosidase on a DEAE-cellulose column (350 x 25 mm). A gradient elution was provided by acetate buffer, pH 4.2 (0.3 to 0.6 M). The eluate was examined for protein (——) and amyloglucosidase(•¯) by spectrophotometry at 258 nm and the DNSA method, respectively. Amyloglucosidase activity was coincident with a single peak eluted between 0.35 and 0.45M acetate buffer.

4.1.3.2. - STEPWISE ELUTION.

Ion exchange preparation:

Pre-cycling: DEAE-cellulose DE-32 (30g) was stirred into 750ml 0.5M HCl and left for 30 min. Subsequently it was washed in a funnel fitted to a Buchner flask under vacuum until the effluent was pH 4.0. Then the ion exchange was resuspended in 750 ml 0.5M NaOH and again left for 30 min, followed by washing as above until the effluent pH was 7.0.

Degassing: The pre-cycled DEAE-cellulose and about 250 ml 0.2M acetic acid were stirred magnetically in a stoppered Buchner flask under vacuum to degass.

Equilibration: The pre-cycled and degassed DEAE-cellulose was introduced into a column (500 x 25 mm) and washed with 0.02M degassed acetate buffer, pH 4.2 until equilibrium was reached.

Application of sample:

An ethanolic precipitation of the crude enzyme preparation of Rhizopus delemar fermentation resuspended in 0.02M acetate buffer, pH 4.2, was applied on the top of the DEAE-cellulose column above described and a stepwise elution carried out with acetate buffer 0.02M pH4.2, 0.2M pH 4.2 and 0.2M pH 5.4.

The flow rate was 3.5ml/min. The effluent stream was analysed, as previously described, in an LKB-Uvicord. The elution volume of the peaks was dialysed against distilled water overnight at 4° and concentrated in vacuo. Amyloglucosidase activity and protein content of these peaks were measured by Lloyd and Whelan's method and the procedure of Lowry et al. respectively.

A typical pattern of the chromatographic behaviour of amyloglucosidase from Rhizopus delemar on DEAE-cellulose DE-32, following these procedures, is shown in Fig. 4.1.3.2.1. This elution profile was obtained by using 4.0ml of an ethanolic precipitate containing 1.223 units.ml^{-1} and 5.0 mg protein.ml^{-1}

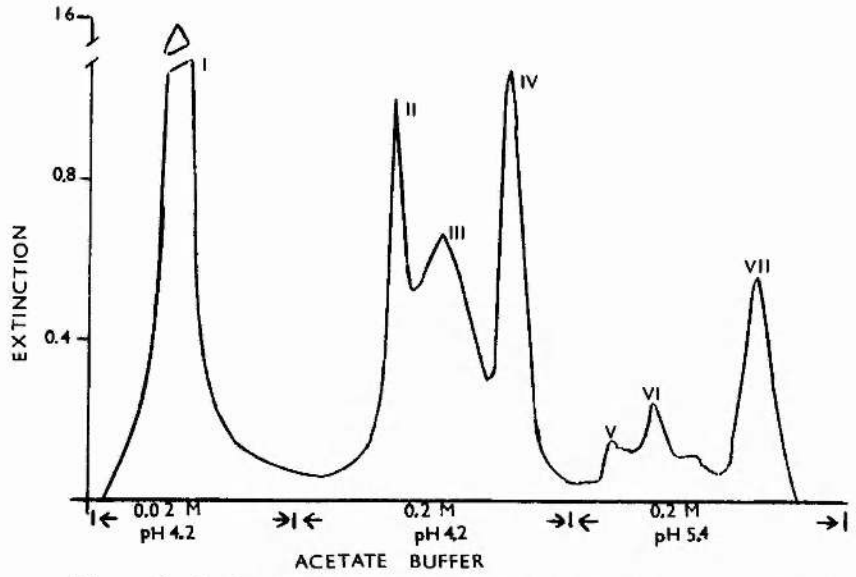


Fig. 4.1.3.2.1. Chromatography of ethanolic precipitation of crude preparation of Rhizopus delemar amyloglucosidase on a DEAE-cellulose column (500 x 25 mm). Stepwise elution was performed as indicated in the chromatogram. The eluate was examined for protein by spectrophotometry at 258 nm and amyloglucosidase activity determined by Lloyd and Whelan's method in the peaks collected. Amyloglucosidase was eluted with the first peak.

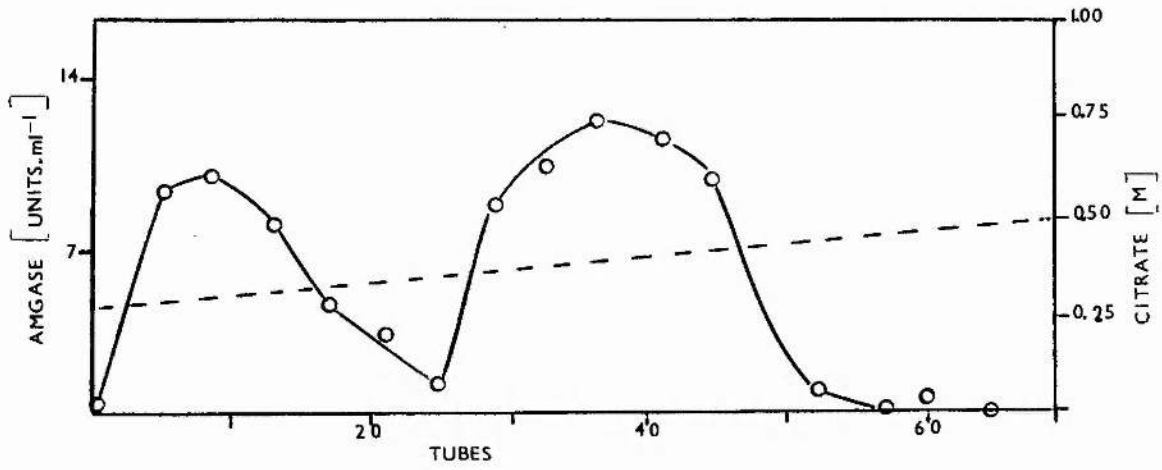


Fig. 4.1.4.1. – DEAE-SEPHADEX A-50 CHROMATOGRAPHY OF CRUDE PREPARATION OF RHIZOPUS DELEMAR AMYLOGLUCOSIDASE. The column was 300 x 15 mm and was eluted with a linear gradient of citrate buffer provided by a Technicon Autograd. The collected fractions were examined for amyloglucosidase activity. Amyloglucosidase was eluted in the second peak.

The amyloglucosidase activity was coincident with the first peak. The material obtained showed a specific activity of 1.42 units. $mg\ protein^{-1}$. The combination of this procedure and the ethanolic precipitation gave a purification of about 15-fold.

This preparation released a maximum of 28% glucose residues from amylose azure, but no soluble coloured products were detected after exhaustive hydrolysis, confirming the absence of traces of α-amylase.

4.1.4. - CHROMATOGRAPHY ON DIETHYLAMINOETHYL-SEPHADEX A-50.

100 mg freeze-dried crude enzyme preparation from the fermentation of Rhizopus delemar in 4 ml 10 mM citrate buffer, pH 6.0 were applied on the top of a column (150 x 30 mm) of DEAE-Sephadex A-50 pre-equilibrated with the same buffer used as a solvent for the sample. A gradient elution was started by using a Technicon Autograd (Technicon Corp., U.S.A.) so that the citrate gradient was linear. Fifty fractions were collected at a flow rate of 60 ml/h. Enzymatic activity was assayed in the fractions by measuring the glucose released according to the reducing method neocuproine.

A representative elution-profile is shown in Fig. 4.1.4.1. and a summary of the results is presented in Table 4.1.4.1. Fig. 4.1.4.2. shows a progress curve for the hydrolysis of 5% oxidised amylose using the preparations obtained.

As seen in Fig. 4.1.4.2., most amyloglucosidase activity was coincident with the second peak, which presented a higher rate of glucose liberation from 5% oxidised amylose than the first peak. Furthermore, the second peak was unable to hydrolyze completely the partially oxidised amylose until an aliquot from the first peak was added. The latter observation also demonstrates that this amyloglucosidase preparation was free of traces of α-amylase, and α-amylase contamination contained in the crude preparation was eluted in the first peak. However, modifications in the properties of the DEAE-sephadex with the ionic strength of the buffer have proved to be a disadvantage of this purification procedure compared with that described in 4.1.3.2.

Table 4.1.4.1. - Purification of *Rhizopus delemar* amyloglucosidase on DEAE-Sephadex A-50.

peak*	Enzymatic Activity (units.ml^{-1})	Protein (mg protein.ml^{-1})	Specific Activity (units.mg protein^{-1})
1st	0.5	0.64	0.78
2nd	0.6	0.47	1.28

* - Obtained as shown in Fig. 4.1.4.1.

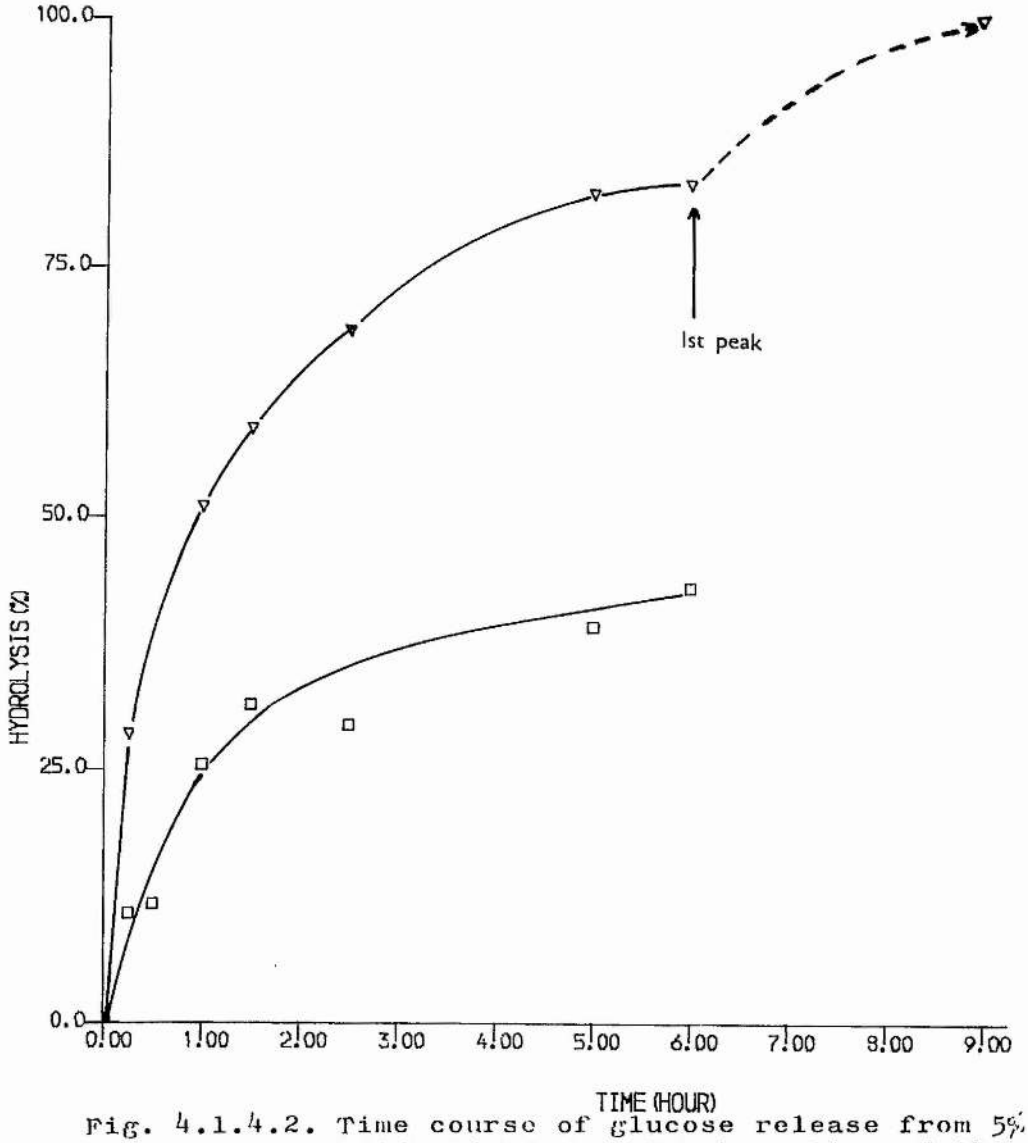


Fig. 4.1.4.2. Time course of glucose release from 5% oxidised amylose by the action of the first (□) and the second (▽) peaks obtained from the purification of Rhizopus delemar amyloglucosidase on DEAE-sephadex A-50. The arrow indicates the addition of an aliquot from the first peak in the second peak - 5% oxidised amylose reaction mixture.

4.1.5. - CHROMATOGRAPHY ON SEPHADEX G-50 AND AMBERLITE IRC-50.

Another method to purify amyloglucosidase preparations was proposed by Pazur & Okada (9) as follows:

Stage I:

A sample of the freeze-dried crude enzyme preparation (8 ml of 100 mg/ml) was applied to a column (280 x 30 mm) of Sephadex G-50 equilibrated in 20 mM sodium acetate buffer, pH 5.0. Twenty five fractions of 2.5 ml were collected at a flow rate of 60 ml/h. Fractions were assayed for amyloglucosidase activity by the DNSA method. Protein content was determined by Warburg & Christian's method.

The fractions containing the bulk of enzymatic activity (8-14th) were pooled and used in Stage II.

Stage II:

The pooled fractions were applied to a column (300 x 30 mm) of Amberlite IRC-50 (cation exchange resin) pre-equilibrated with 20 mM sodium acetate buffer, pH 5.0. Fifteen fractions of 2.5 ml were collected at a flow rate of 80 ml/h. Enzyme activity and protein content were determined as in Stage I. Amyloglucosidase was found in the fractions 9 and 10.

The data from a purification of amyloglucosidase by this procedure is summarized in Table 4.1.5.1. Fig. 4.1.5.1. shows a progress curve for the hydrolysis of 5% oxidised amylose using this preparation. As seen in this figure, amyloglucosidase purified by the present procedure is unable to hydrolyze completely the partially oxidised amylose, except when α-amylase is added to the incubation mixture. This proves that this method of amyloglucosidase purification removes traces of α-amylase from the enzyme preparation. The use of two chromatographies emerged as a disadvantage compared with the purification procedure described in 4.1.3.2.

TABLE 4.1.5.1. - PURIFICATION OF AMYLOGLUCOSIDASE FROM RHIZOPUS DELEMAR BY CHROMATOGRAPHY ON SEPHADEX G-50 AND AMBERLITE IRC-50.

Preparation	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg prot.)	Yield overall (%)	Fold purification overall
Crude enzyme	8	40	1.217	0.030	100	1
Sephadex G-50 and Amberlite IRC-50 Chromatographies.	5	0.24	0.277	1.154	22.7	38.5

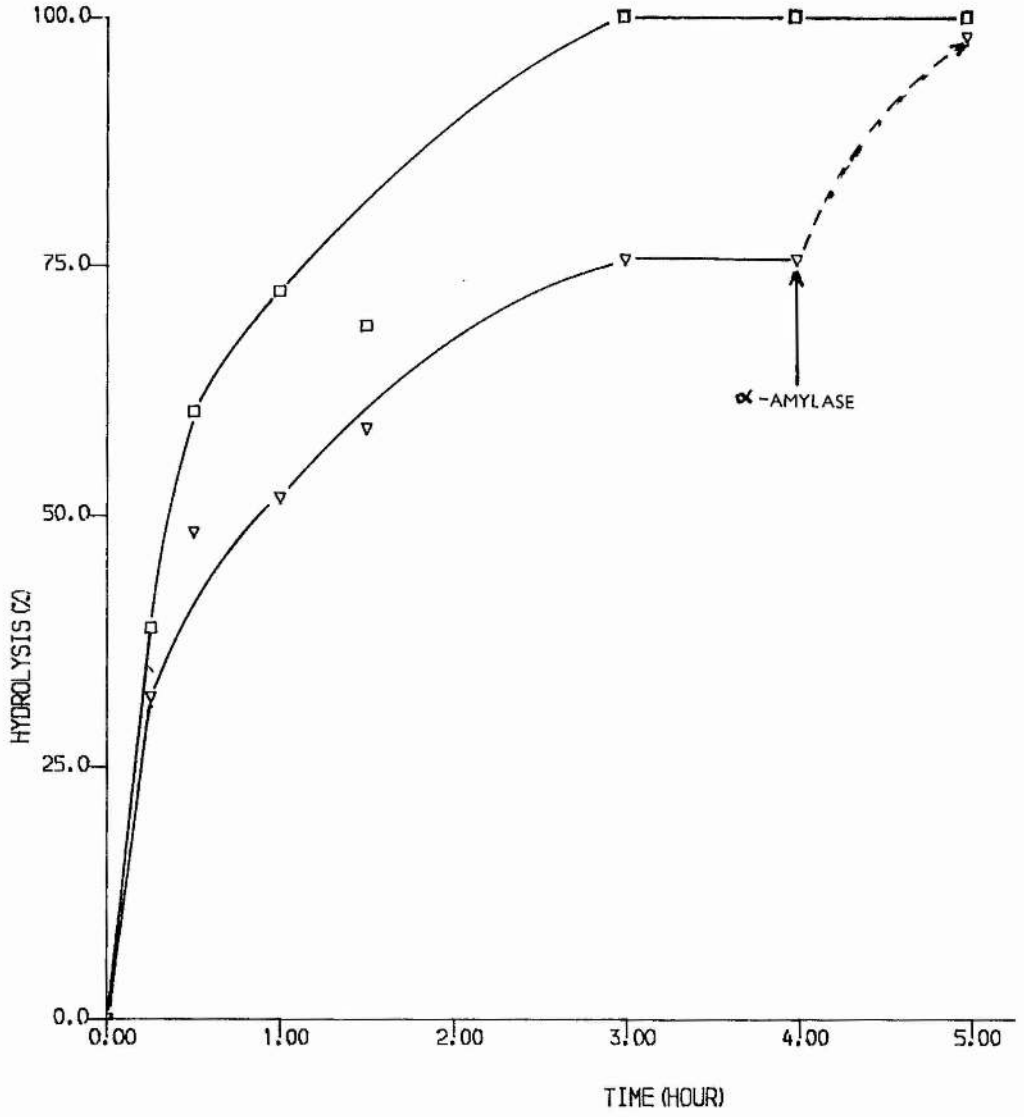


Fig. 1.1.5.1. Time course of action of amyloglucosidase purified on Sephadex G-50/Amberlite on amylose (□) and 5% oxidised amylose (▽). The arrow indicates the addition of ∝-amylase in the 5% oxidised amylose-amyloglucosidase reaction mixture.

4.2. - AMYLOGLUCOSIDASE ACTION ON REMAZOL BRILLIANT BLUE DERIVATIVES OF α -1,4-GLUCANS.

Experiments were carried out to investigate the amyloglucosidase action on Remazol Brilliant Blue (RBB) derivatives of α-1,4-glucans.

The enzyme used was one which has been purified according to the procedure related in 4.1.5. and the absence of traces of α-amylase has been proved following Marshall and Whelan's method described in 3.6.1.

The derivatives were RBB-amylose (amylose azure) and RBB-amylopectin (amylopectin azure).

The detection of glucose and/or a coloured product released by the enzyme activity was performed as described in 3.3.3. (Lloyd and Whelan's method) and 3.6.2., respectively.

The results of these investigations are presented in Fig. 4.2.1. and Fig. 4.2.2.

As reported by Marshall (35) for Cibachron Blue amylose, amyloglucosidase is also unable to release coloured products from Remazol Brilliant Blue derivatives of α -1,4-glucans although it can liberate a limited amount of glucose.

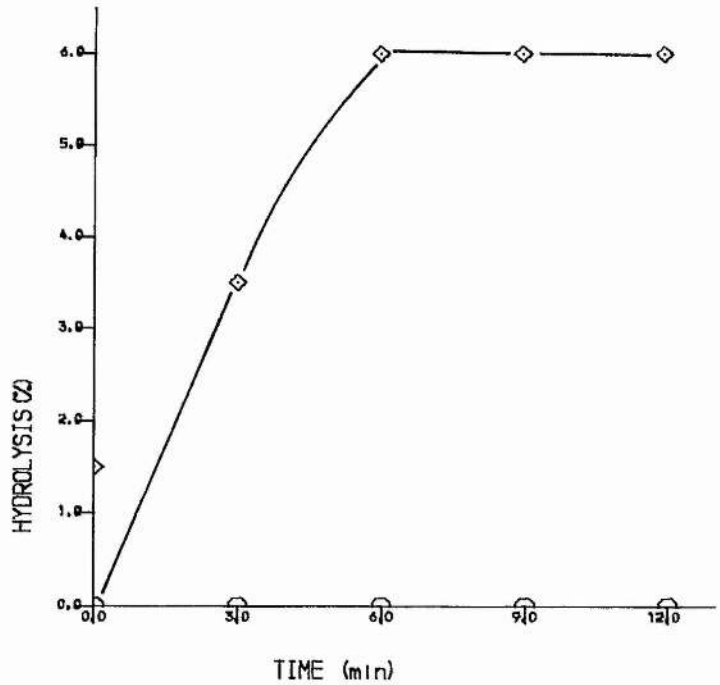


Fig. 4.2.1. – Time course of glucose( ) and coloured product liberation( ) from Remazol Brilliant Blue derivative of amylose(amylose azure) by the action of amyloglucosidase free of traces of ∝ -amylase.

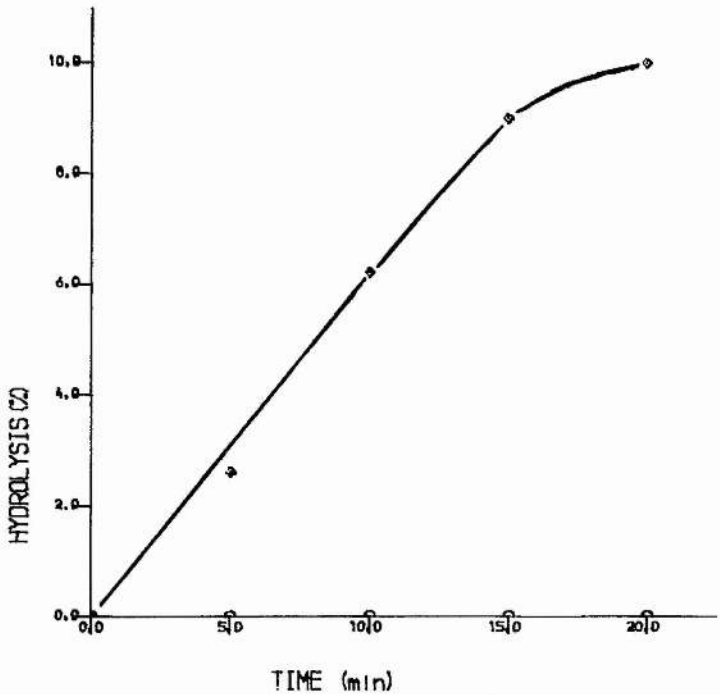


Fig.4.2.2. – Time course of glucose( ) and coloured product liberation( )from Remazol Brilliant Blue derivative of amylopectin(amylopectin azure) by the action of amyloglucosidase free of traces of ∝ -amylase.

4.3. - AMYLOGLUCOSIDASE ACTION IN THE PRESENCE OF α-AMYLASE.

The effects of traces of α-amylase contamination in amyloglucosidase preparations have been considered so far in relation to the detection of such contamination using modified substrates. The effect of this endoamylolytic enzyme on the kinetics of amyloglucosidase has also been investigated.

Attempts to study quantitatively the kinetics of amyloglucosidase action in the presence of α-amylase will involve complicated equations unless some simplifications are introduced.

Sawicki (72) has suggested a kinetic model for the simultaneous hydrolysis of starch by α-amylase, β-amylase and maltase. A complicated equation was found to describe the rate of glucose release with time. However, the author has no experimental evidence for his model.

In this work, the following theoretical and experimental approach was developed to investigate the effect of α-amylase on amyloglucosidase kinetics:

Initially, it is necessary to consider simply amyloglucosidase kinetics. It is known that the apparent kinetic parameters of amyloglucosidase vary with the size of the substrate employed. In other words, the increase in DP of substrate results in a decrease in V and Km, at least with DP $>$ 7.

Thoma & Koshland (31) have proposed an explanation for this decrease in Km and V based on competitive inhibition by internal residues of the α-1,4-glucans. They pointed out that such inhibition would not be observable using the classical equations since the concentration of inhibitor will be proportional to the substrate.

The classical equation describing competitive inhibition can be represented by the formula:

$$v = \frac{VS}{Km\left(1 + \frac{I}{Ki}\right) + S} \quad (1)$$

For α-1,4-glucans of n monomers complexed by amyloglucosidase there will be one active and (n-m) inactive complexes, where m represents the number of glucose units over which the binding site is extended (m = 2). The concentration of internal residues which causes inhibition is given by (n-m)S. Then equation 1 will be

$$v = \frac{VS}{Km\left[1 + \frac{(n-m)S}{Ki}\right] + S} \quad (2)$$

It is worthwhile to note that equation 2 is homeomorphic with Michaelis Menten equation, provided Km' and V' (apparent Michaelis constant and maximum velocity) are interpreted as

$$V' = V\left[\frac{1}{1 + (n-m)(Km/Ki)}\right] \quad (3)$$

$$Km' = Km\left[\frac{1}{1 + (n-m)(Km/Ki)}\right] \quad (4)$$

Equations 3 and 4 predict the decrease in V and Km as the DP of substrate increases, since higher values for (n-m) result in lower values for the expression in brackets.

While equation 2 is in fact oversimplified (single chain attack and competitive inhibition by product being ignored) it permits the investigation of the simultaneous action of α-amylase with amyloglucosidase.

Since α-amylase is an endoamylase there is a rapid decrease in the average molecular weight of the substrate con-

trasting with the action of an exo enzyme such as amyloglucosidase. This effect of α-amylase which has been termed "dextrinization" is observed as a first phase of α-amylase action when the intensity of blue value (iodine staining power) and the viscosity rapidly decrease.

As stated above, the kinetic parameters (Km and V) of amyloglucosidase depend on the average molecular weight of substrate, hence rapid reduction in the average molecular weight caused by the presence of α-amylase will naturally affect these observed parameters.

Fig. 4.3.1. shows the time course of glucose release from α-1,4-glucan (amylose) by amyloglucosidase, free of traces of α-amylase (purified as described in 4.1.3.2.), and the effect of subsequent addition of α-amylase at the point indicated. Whereas Fig. 4.3.2. shows the time course of glucose release firstly only with α-amylase followed by the addition of amyloglucosidase at the point indicated. These experiments clearly show that the increased rate of glucose release in Fig. 4.3.1. after addition of α-amylase could not have been due to the action of α-amylase per se.

Fig. 4.3.3 presents the time course of the decrease in average molecular weight of amylose ($\overline{DP}$ = 797) upon α-amylase action. The procedure for determining molecular weight of amylose, based on method of Hiromi et al. (73), was carried out as follows:

Amylose and α-amylase were incubated at 40° and aliquots of the reaction mixture were withdrawn at appropriate time intervals and immediately incubated at 100° to denature α-amylase. These samples were cooled in ice and incubated in the reaction vessel of an oxygen electrode at 40°. Amyloglucosidase, free of traces of α-amylase (purified as in 4.1.3.2), was introduced and

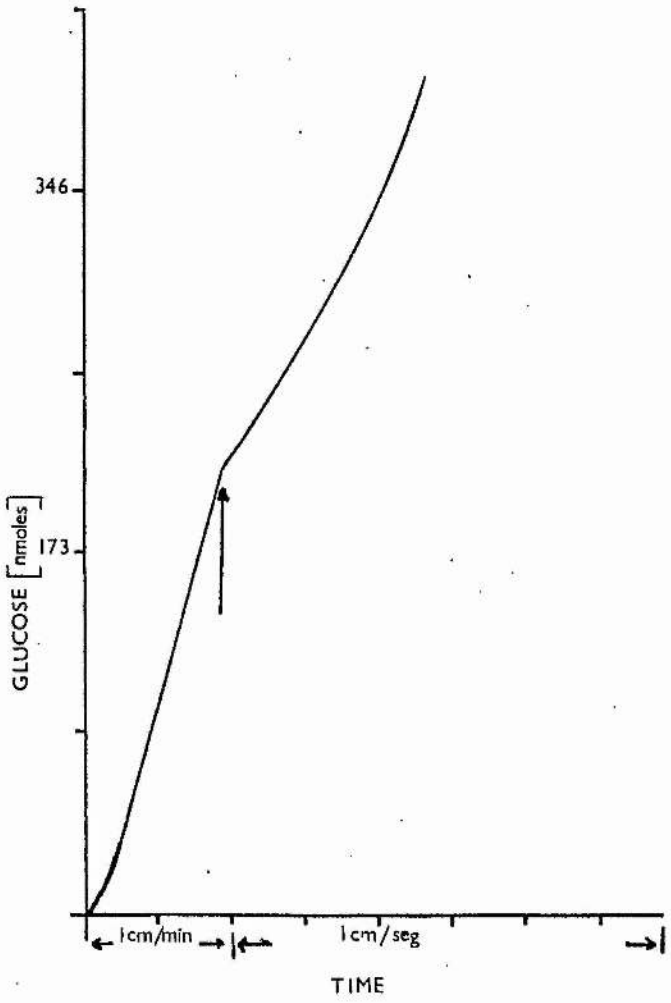


Fig. 4.3.1. - Time course of glucose release from amylose by the action of amyloglucosidase free of traces of α-amylase. The arrow indicates the addition of 200 μg α-amylase. The reaction mixture was composed of 38 μM amylose, 60 μg glucose oxidase, 100 μg of amyloglucosidase and 50 mM phosphate/citrate buffer, pH 5.0 (Vol. = 2.00, T = 40°). Glucose was determined by the glucose oxidase/ oxygen electrode method.

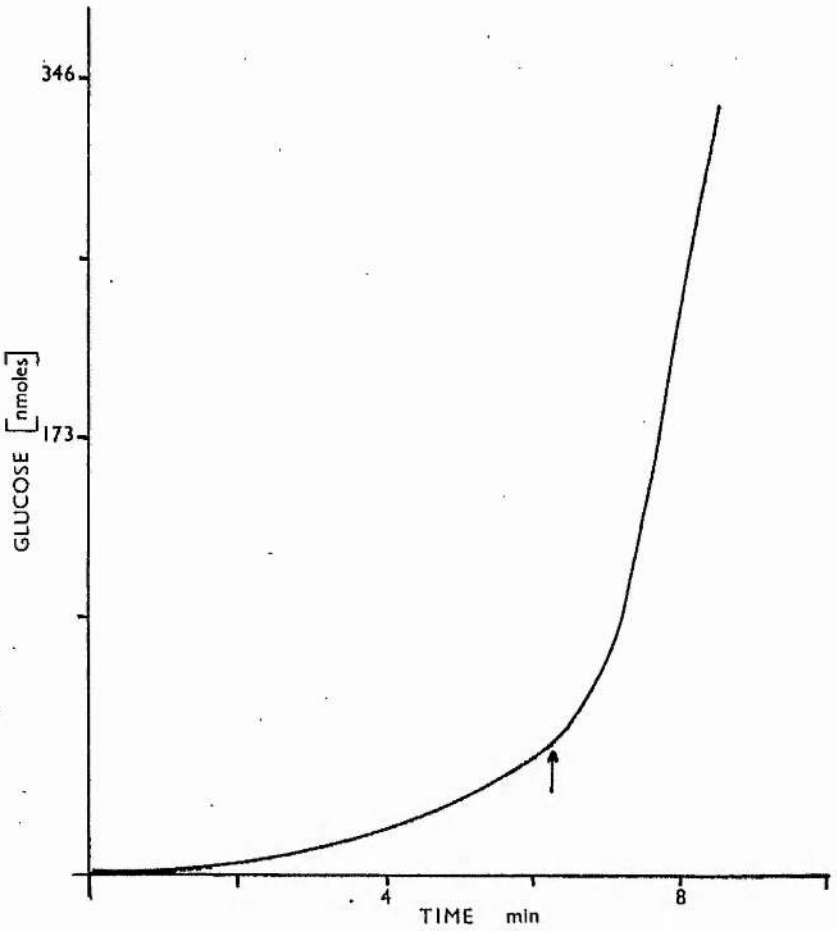


Fig. 4.3.2. - Time course of glucose release from amylose by the action of α-amylase. The arrow indicates the addition of 100 μg of amyloglucosidase free of traces of α-amylase. The reaction mixture was composed of 38 μM amylose, 60 μg glucose oxidase, 200 μg α-amylase and 50 mM phosphate/citrate buffer, pH 5.0 (Vol. = 3.00 ml, T = 40°).

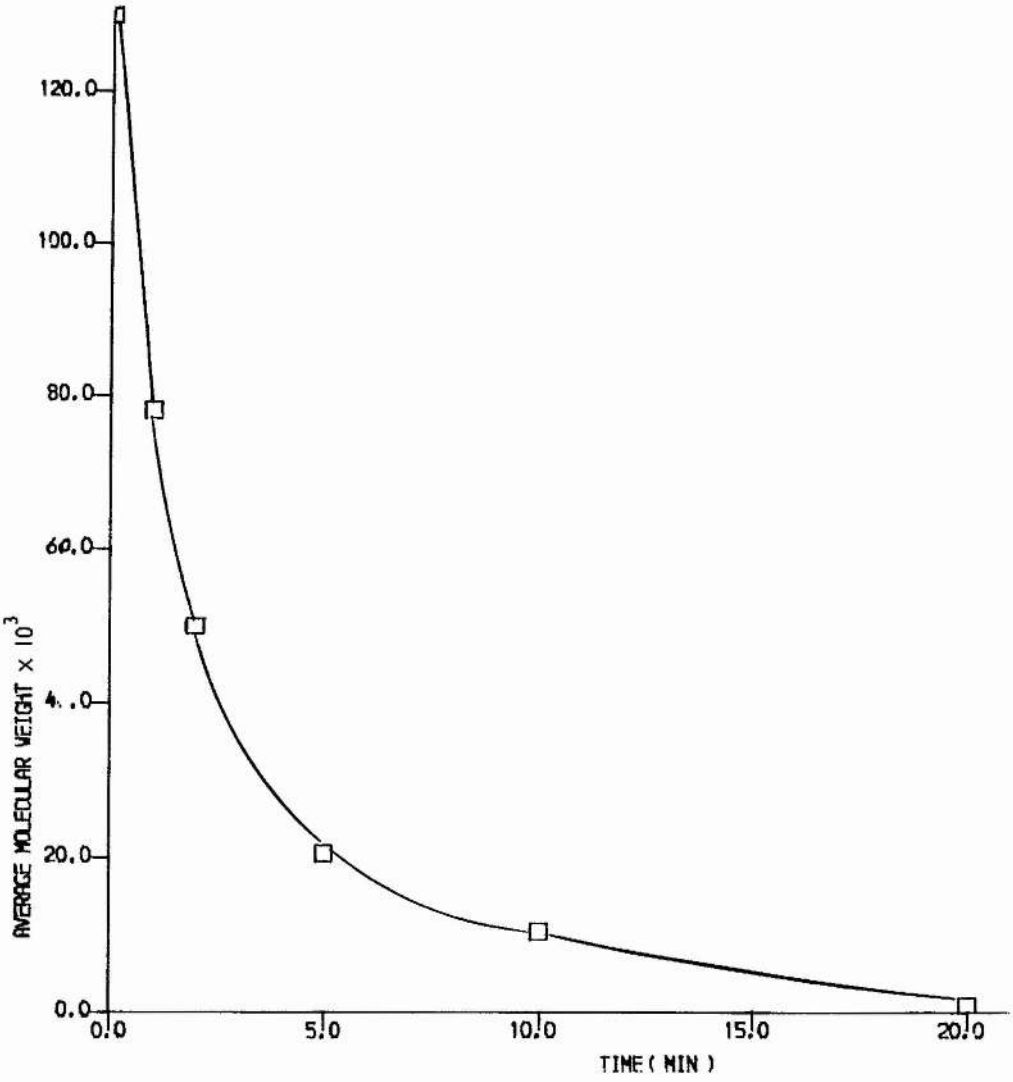


Fig. 4.3.3. - Time course of the decrease of the average molecular weight of amylose by the action of ∝-amylase. The determination of the average molecular weight of the samples removed from an amylose ∝-amylase reaction mixture was performed according to Hiromi et al's procedure (73).

the rate of glucose released followed by glucose oxidase/oxygen electrode procedure. From the plot of time/glucose versus glucose, the slope s was obtained. The average molecular weight was determined according to the following equation:

$$\overline{\text{Mol.wt}}_t = \overline{\text{Mol.wt}}_o \times s/s_o \quad (5)$$

where

$\overline{\text{Mol.wt.}}_t$:- Average molecular weight of substrate removed at t time of amylose/α-amylase incubation mixture.

$\overline{\text{Mol.wt.}}_o$:- Average molecular weight of amylose. In this particular case a value of 130,000 was used since an amylose with a DP = 797 was employed.

s :- Slope from time/glucose versus glucose plot for the aliquot at t time.

s_o :- Slope from time/glucose versus glucose plot for the amylose.

Fig. 4.3.4. shows a s/v against s plot of amyloglucosidase action on amylose (DP = 797) in the presence and absence of α-amylase. The values of apparent Km (23.01 ± 1.29 μM) and apparent V(175.40 ± 4.00 nmoles.min^{-1}) for amyloglucosidase in the presence of α-amylase are greater than those (10.77 ± 0.54 μM and 87.87 ± 1.19nmoles.min^{-1}) found for pure amyloglucosidase.

The advantage of adding α-amylase to α-1,4-glucan/amyloglucosidase systems, as far as the hydrolysis of such polymers by the latter enzyme to produce glucose is concerned, is exploited in nature since both enzymes are simultaneously released by many organisms including the fungal species Rhizopus and Aspergillum.

The presence of α-amylase and amyloglucosidase acting together resulting in the production of higher amounts of glucose in shorter time intervals will hold considerable commercial interest

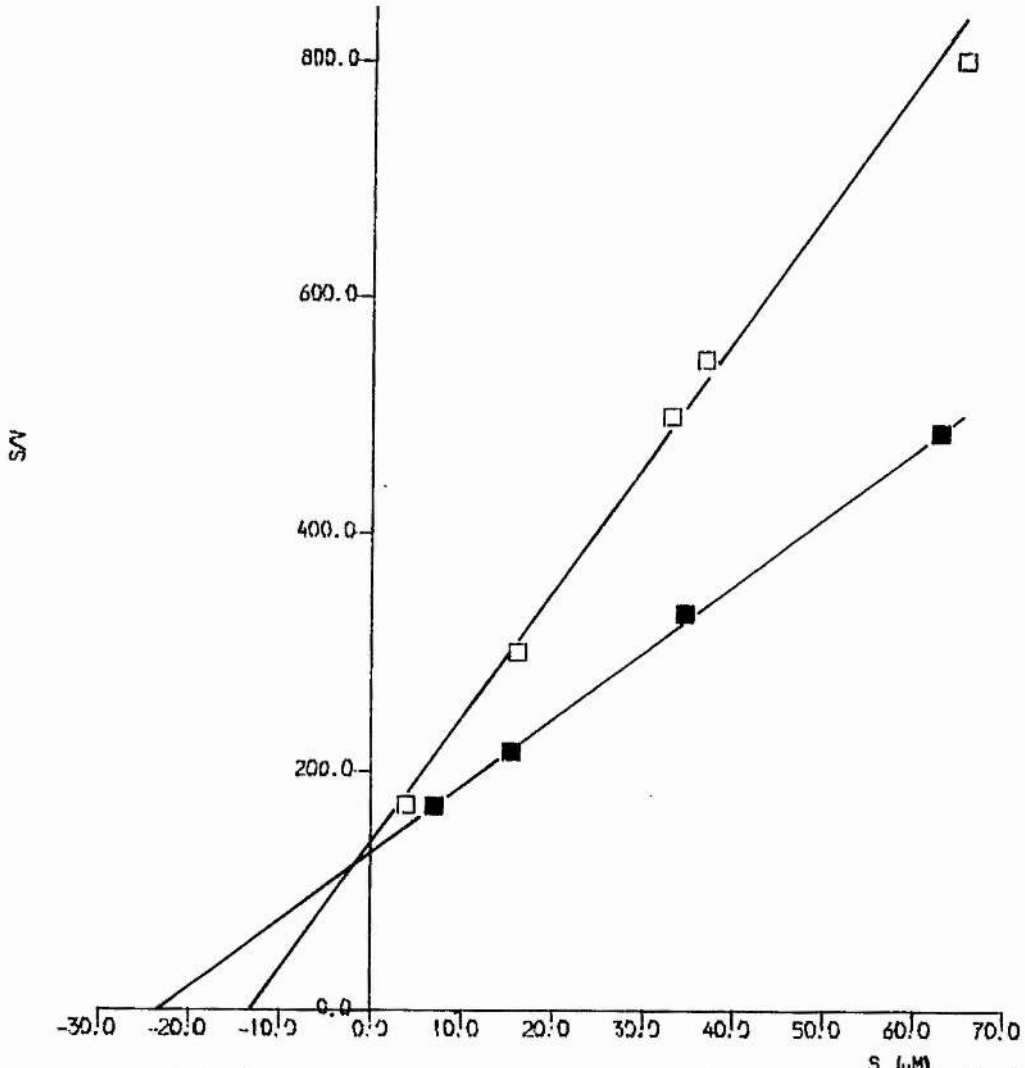


Fig. 4.3.4. - Comparison between s/v versus s plot for amyloglucosidase catalysed hydrolysis of amylose in the absence (□) and in the presence (■) of α-amylase. pH 5.0, T = 40°. Amyloglucosidase and α-amylase concentration were 50 $\mu g.ml^{-1}$ and 100 $\mu g.ml^{-1}$, respectively. s/v is expressed in 10^3 x $\mu M.nmoles^{-1}.min$.

4.4. - AMYLOGLUCOSIDASE ACTION ON OXIDISED AMYLOSE.

Amyloglucosidase activity is blocked by the presence of oxidation points in the α-1,4-glucans substrates. The kinetics of this inhibition have been investigated.

i) Theory:

In amyloglucosidase catalysed hydrolysis of partial oxidised linear α-1,4-glucan substrate, the enzyme-substrate complex formation faces two possibilities; an active complex of a non-reducing unit with amyloglucosidase and an inactive complex of an oxidised non-reducing unit with amyloglucosidase. The latter occurrence will lead to competitive inhibition. Therefore, the reaction schemes and the rate law can be represented as follows:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \qquad (1)$$

$$E + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EI \qquad (2)$$

$$Km = \frac{k_{-1} + k_{+2}}{k_{+1}} \qquad (3)$$

$$Ki = \frac{k_{-3}}{k_{+3}} \qquad (4)$$

$$\frac{1}{v} = \frac{1}{V}\left[1 + Km/S \ (1 + I/Ki)\right] \qquad (5)$$

However, this kind of inhibition can not be demonstrated with these conventional equations since the concentration of inhibitor is directly proportional to the concentration of substrate. In this sense, the competitive inhibition by partially

oxidised substrate molecules resembles competitive inhibition by internal residues of substrate as studied by Thoma & Koshland(31)

Consider a linear polymer consisting of a number n of residues, in which p are oxidised. Thus , for any polymer complexed to amyloglucosidase there will be (n-p) active and p inactive complexes. Then the concentration of inhibitor is given by the following equations:

$$I = \left(\frac{p}{n-p}\right)S \qquad (6)$$

and

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{Km}{S} + \left(\frac{p}{n-p} \cdot \frac{Km}{Ki}\right)\right] \qquad (7)$$

Equation 7 can be simplified into the linear form of Michaelis-Menten equation,

$$\frac{1}{v} = \frac{1}{V'}\left(1 + Km'/S\right) \qquad (8)$$

in which V' (observed maximum velocity) and Km' (observed Michaelis - constant) are complex functions given by the following equations:

$$V' = V\left[\frac{1}{1 + \frac{p}{n-p} \cdot \frac{Km}{Ki}}\right] \qquad (9)$$

and

$$Km' = Km\left[\frac{1}{1 + \frac{p}{n-p} \cdot \frac{Km}{Ki}}\right] \qquad (10)$$

Equations 9 and 10 can also be written in the following inverse forms:

$$\frac{1}{V'} = \frac{1}{V} + \frac{Km/Ki}{V} \cdot \frac{p}{n-p} \qquad (11)$$

$$\frac{1}{Km'} = \frac{1}{Km} + \frac{1}{Ki} \cdot \frac{p}{n-p} \qquad (12)$$

Equations 11 and 12 predict that plots of 1/V' and 1/Km' versus (p/(n-p)) will be straight lines and in the latter case the slope will be 1/Ki. Therefore, these observations provide an opportunity to test this theory experimentally.

In addition, if p is equal to zero (no oxidation) the observed Michaelis constant Km' will be reduced to Km, namely, the Michaelis constant in the absence of oxidation of the substrate. The same consideration will apply to the observed maximum velocity.

On the other hand, if 100% oxidation of substrate is carried out, p is equal to n, the observed Michaelis constant and the observed maximum velocity will be equal to zero.

ii) Experimental:

The amyloglucosidase action on oxidised amylose was investigated by employing 15,30,45 and 60% oxidised amyloses and an enzyme preparation purified on DEAE-cellulose which was free of traces of α-amylase according to the chromogenic substrate test procedure. The study in the absence of oxidation was performed with unoxidised amylose ($\overline{DP}$=797) from the same origin as the oxidised preparations. The enzymatic activity was measured by the ascorbic acid method as described in 3.4.1., except that the chart speed was decreased to 1 cm/5 min.

Fig. 4.4.1. shows the glucose released from unoxidised and oxidised amyloses by the action of amyloglucosidase at 40°, pH 5.8, when a concentration of 0.166% (w/v) of substrate was used.

Initial velocities were directly estimated from the initial slope obtained from reaction-time curves such as the typical examples in Fig. 4.4.1.

In Fig. 4.4.2. are shown the s/v versus s plots for each substrate. The values of Km and V and their relevant standard errors are listed in Table 4.4.1. The Michaelis Constant Km and the maximal velocity V decreased with the increasing oxidation of amylose and those relationships are better displayed in Fig. 4.4.3.

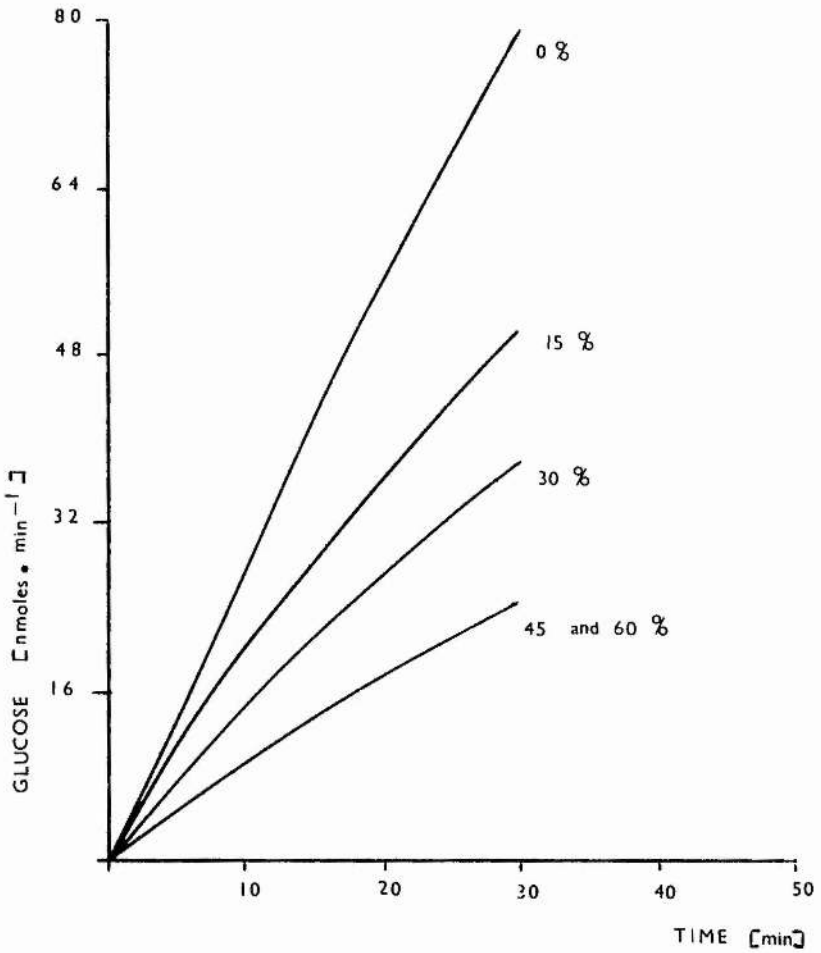


Fig. 3.3.1. Time course of glucose release from unoxidised and oxidised amyloses by amyloglucosidase action.

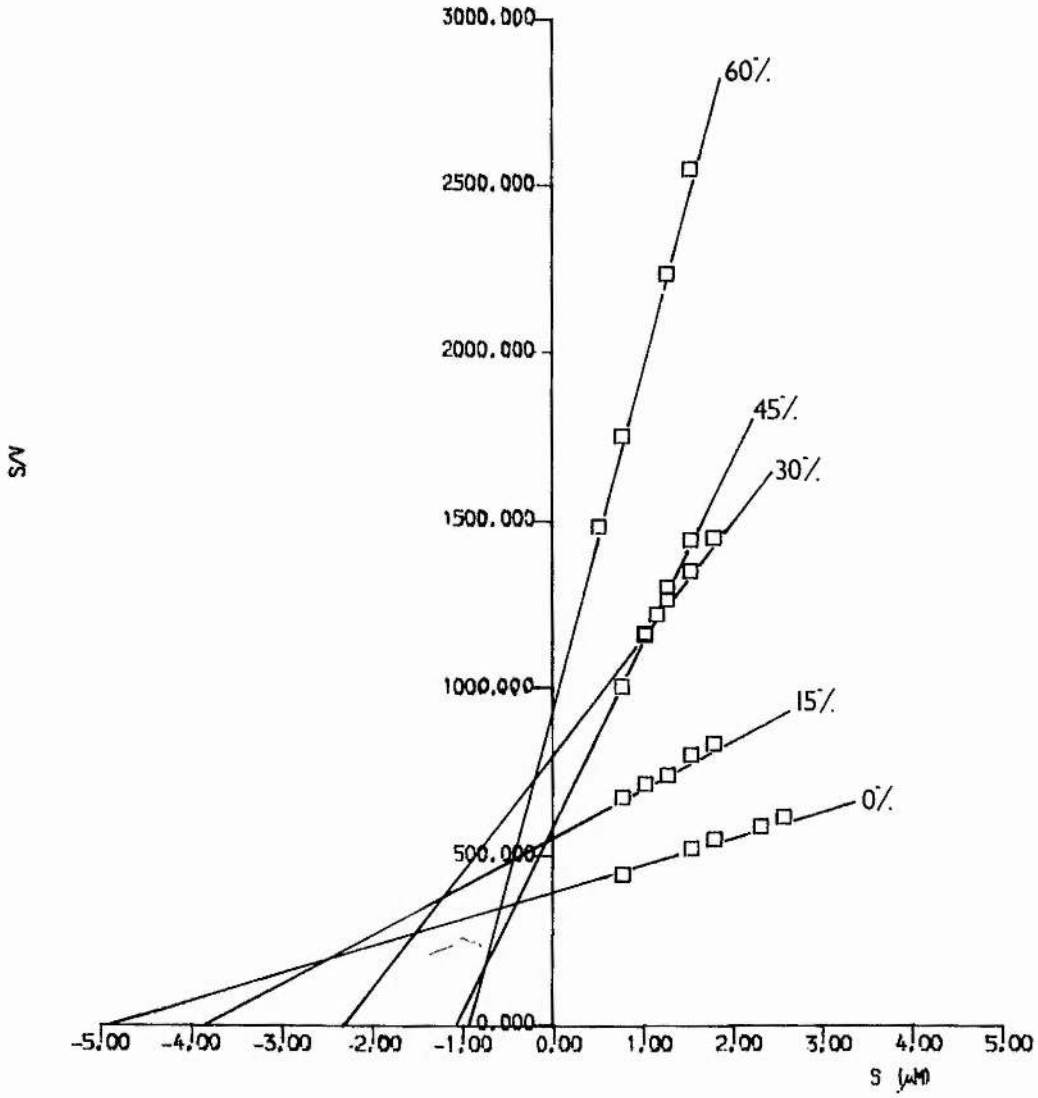
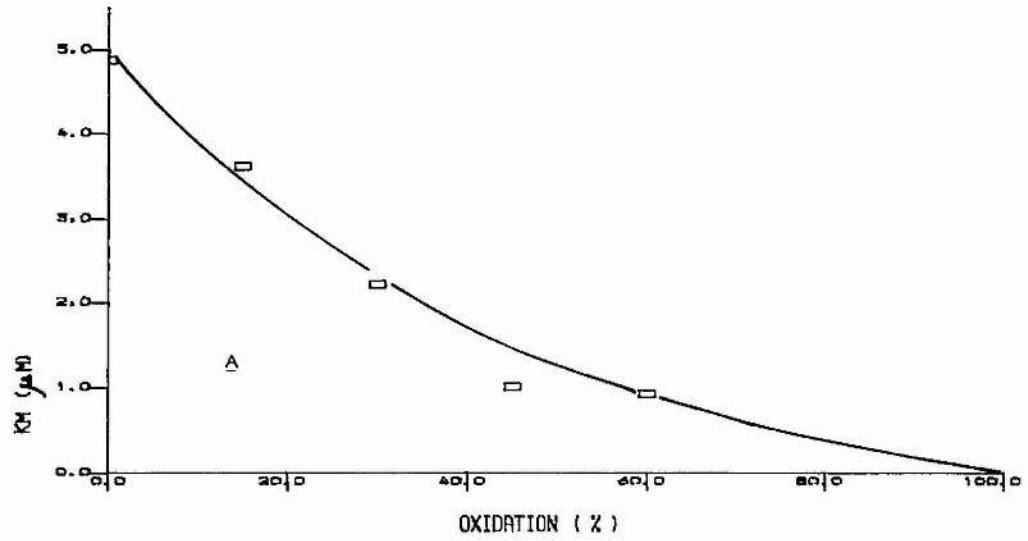


Fig. 4.4.2. - s/v VERSUS s PLOTS FOR AMYLOGLUCOSIDASE-CATALYSED HYDROLYSIS OF UNOXIDISED AND OXIDISED AMYLOSES. pH 5.0, T = 40^{o}, enzyme concentration = 40 nM. s/v is expressed in 10^{3} x µM. $mmoles^{-1}$.min.

TABLE 4.4.1. - THE VALUES OF Km AND V AND THEIR RELEVANT STANDARD ERRORS* FOR AMYLOGLUCOSIDASE ACTION ON UNOXIDISED AND OXIDISED AMYLOSES.

Percentage of oxidation of amylose	Km ± standard error (uM)	V ± standard error (nmoles.min^{-1})
0	4.88 ± 0.27	12.00 ± 1.14
15	3.62 ± 0.19	6.46 ± 0.24
30	2.22 ± 0.14	2.78 ± 0.15
45	1.02 ± 0.05	1.77 ± 0.04
60	0.93 ± 0.06	0.97 ± 0.03

* - Calculated by the method recommended by Wilkinson (80) (See appendix).



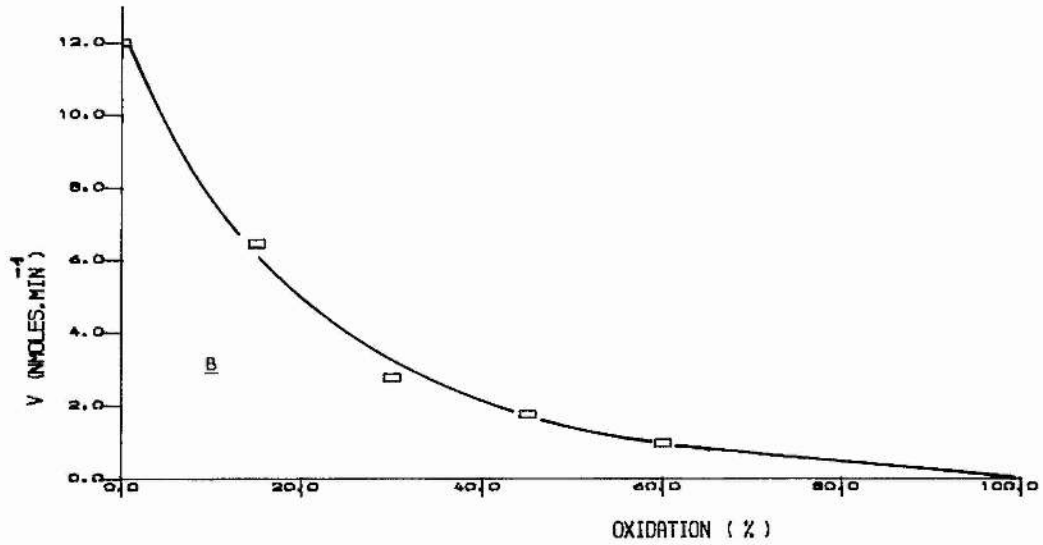


Fig. 4.4.3. A) Dependence of the apparent Km of amyloglucosidase on the degree of oxidation of amylose.

B) Dependence of the apparent V of amyloglucosidase on the degree of oxidation of amylose.

The kinetic features above, obtained with unoxidised and oxidised amyloses, are consistent with those predicted theoretically. Thus, if n is the total number of residues and p the number oxidised, the plots of 1/Km and 1/V obtained against p/(n-p) will be straight lines as shown in Fig. 4.4.4., from which the inhibition constant Ki (the inverse of the slope of the former plot) can be calculated (Ki = 1.02μM).

As stated earlier and demonstrated here, the observed Km and V should attain the value of zero (unmeasurable) when p=n (100% of residues oxidised). Equally, as p tends to zero, Km' and V' will tend to Km and V.

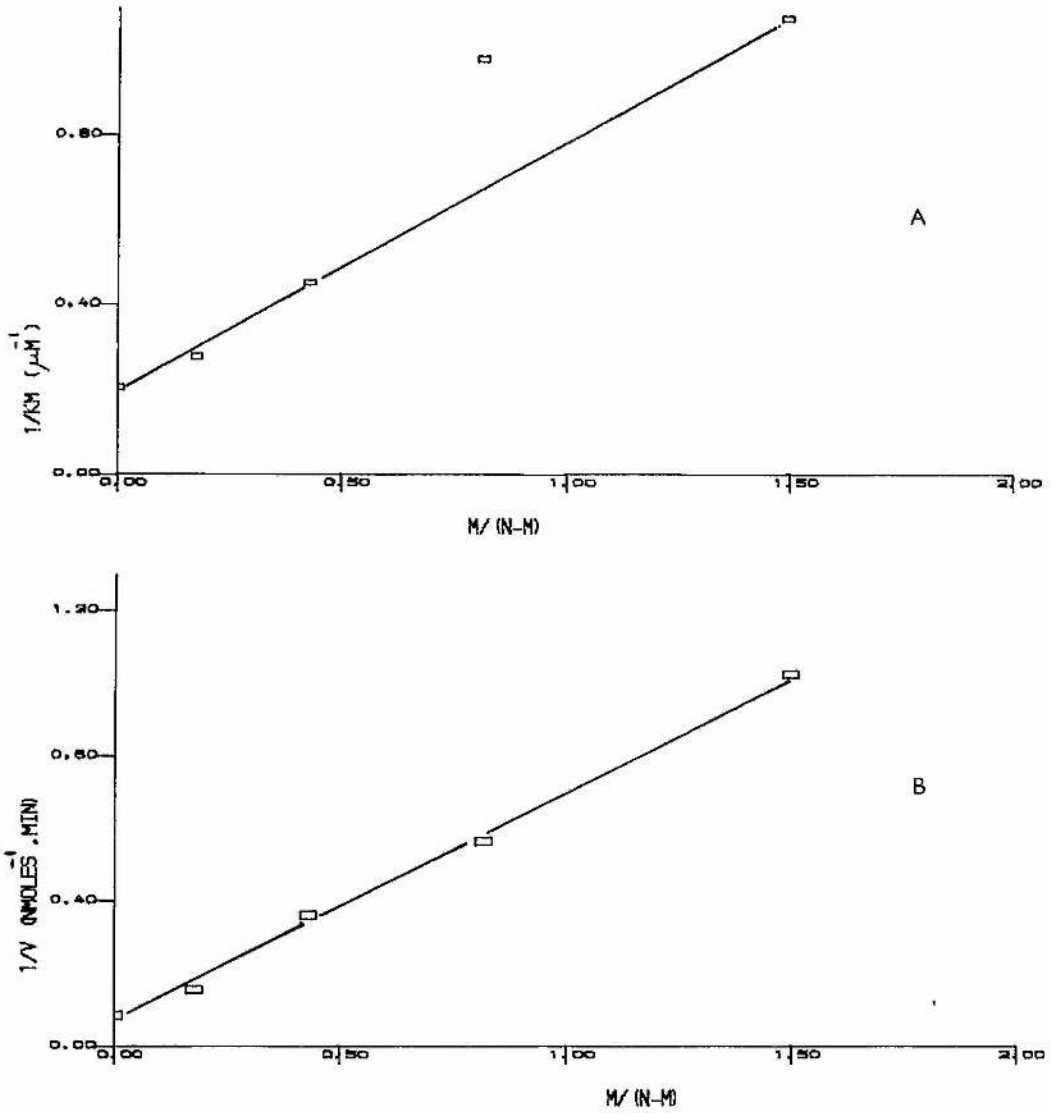


Fig. 4.4.4. A) 1/Km versus m/(n-m) plot for amyloglucosidase-catalysed hydrolysis of unoxidised and oxidised amyloses.
B) 1/V versus m/(n-m) plot for amyloglucosidase-catalysed hydrolysis of unoxidised and oxidised amyloses. n and m stand for the number of glucose units and the number of oxidised glucose units in the amylose molecule, respectively.

4.5. - AMYLOGLUCOSIDASE ACTION ON MALTOSE.

To investigate the kinetics of amyloglucosidase action on maltose as substrate, 0.258 units of enzyme were incubated in varying maltose concentrations (0.2 to 10mM) and the initial velocities calculated by Lloyd and Whelan's method. The assays were carried out in 20 mM sodium acetate buffer, pH 5.0 at 40°.

The present values of 1.95 ± 0.05 mM for Km and 74.99 ± 0.70 mmoles.min^{-1} for V (See Fig. 4.5.1.) for amyloglucosidase on maltose are of the same order of magnitude as those reported by Hiromi *et al.* and Ono *et al.* (30) and different from the value of 6.1 mM reported by O'Neill *et al.* (39) for the same system. It is interesting to note that these latter authors obtained a value of 1.4 mM for the Km for the maltose- DEAE-cellulose amyloglucosidase immobilised system, but could offer no explanation for this fall in Km after immobilisation of the enzyme (as they noted no charge effects and film diffusion resistance effects apply in this system). It may be that the O'Neill *et al.* Km value for the free enzyme is suspect, and that in reality there exists no difference in Km for maltose between the free enzyme and the immobilised enzyme.

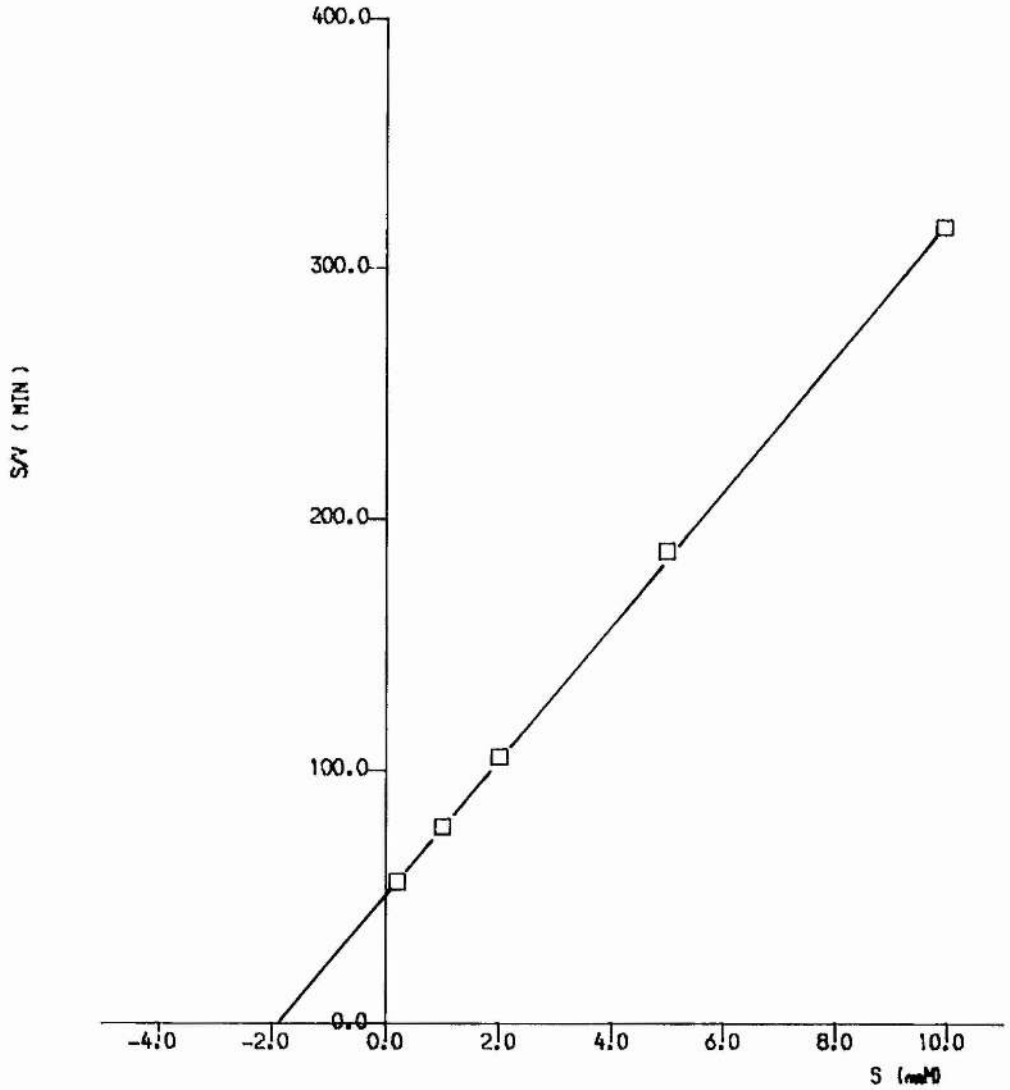


Fig. 4.5.1. s/v versus s plot for amyloglucosidase catalysed hydrolysis of maltose. pH 5.0, T = 40°. Enzyme concentration = 0.14 μM.

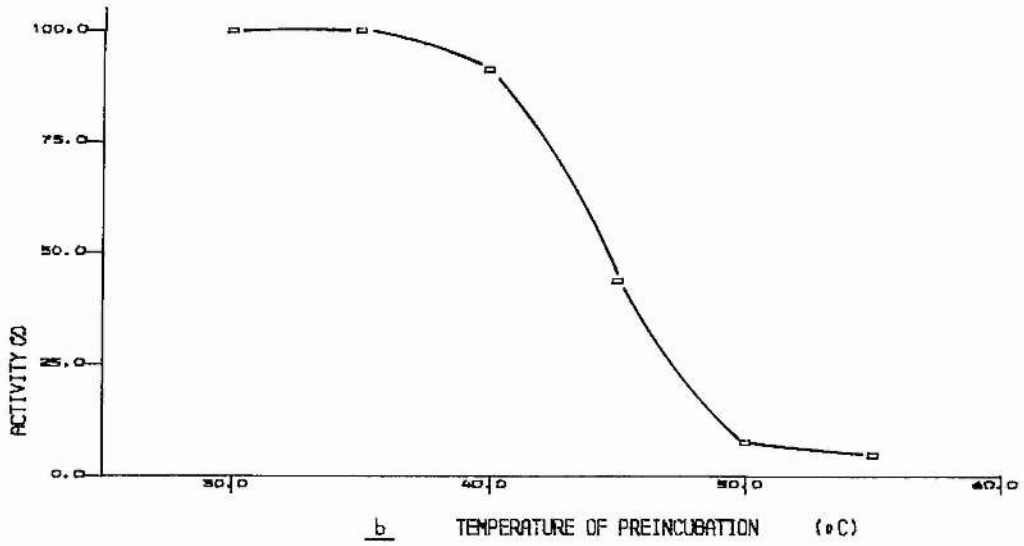
4.6. - pH PROFILE AND THERMAL STABILITY OF AMYLOGLUCOSIDASE.

To investigate the effect of pH on the enzyme activity, 0.258 units of amyloglucosidase were incubated in 0.5 % (w/v) soluble starch at varying pH's provided by 50 mM sodium phosphate/citrate buffer at 40°. The enzyme assay was established by the neocuproine method and the initial velocities worked out from these assays were plotted in terms of percentages (highest value was considered as 100%) against pH (See Fig. 4.6.1.a).

The thermal stability was determined by incubating aliquots of 0.258 units of amyloglucosidase at different temperatures for 30 min, cooling them in ice afterwards and assaying their activities in 0.5% (w/v) soluble starch in 50 mM sodium phosphate/citrate buffer, pH 5.0 by the DNSA method. The percentages of initial velocities retained after these thermal treatments were plotted against their respective temperatures (See Fig. 4.6.1.b).

As seen in Fig. 4.6.1.a, amyloglucosidase has a broad optimum pH ranging from 4.5 to 5.0. This observation is in accordance with the previous report by Phillips & Caldwell (8).

In addition, Fig. 4.6.1.b shows that amyloglucosidase is considerably stable at 40° (temperature used for assaying amyloglucosidase throughout this project). A rapid fall in stability is observed for temperatures of pre-incubation higher than 40°. Unfortunately, there have not been similar experiments of amyloglucosidase thermal stability reported in the literature for comparison.



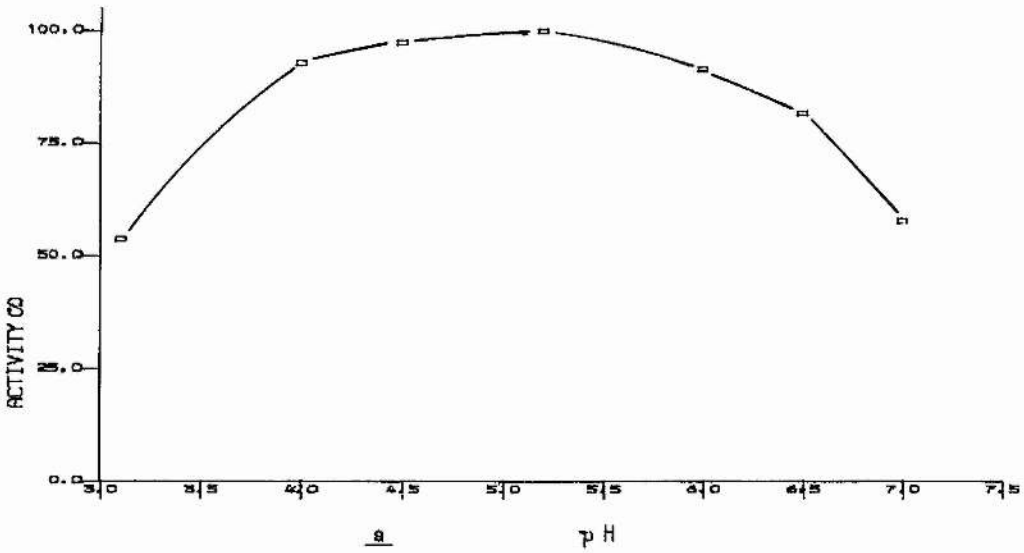


Fig. 4.6.1. a) pH activity profile for Rhizopus delemar amyloglucosidase

b) Thermal stability for Rhizopus delemar amyloglucosidase.

4.7. - EVALUATION OF THE ASCORBIC ACID METHOD.

A method of assaying amyloglucosidase activity has been proposed earlier, in which ascorbic acid replaced o-dianisidine in Huggett & Nixon's method of glucose determination.

This method is based on the following enzyme reactions:

$$\alpha\text{-1,4-glucan} + H_2O \xrightarrow{\text{amyloglucosidase}} \beta\text{-D-glucose} \quad (1)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-}\delta\text{-lactone.} \quad (2)$$

$$H_2O_2 + \text{ascorbic acid} \xrightarrow{\text{peroxidase}} H_2O + \text{dehydroascorbic acid} \quad (3)$$

Here, the merits and limitations of this procedure are discussed. These comments can be summarized as follows:

i) Interference of nucleic acids and proteins.

ii) Rate behaviour of the enzymic reactions.

iii) Comparison against established procedure.

iv) Presence of oxalate.

i) Interference of nucleic acids and proteins.

The absorbance of ascorbic acid at 268 nm and its employment in this assay could be affected by the presence of nucleic acids and proteins which also absorb at this wavelength. However, nucleic acids are not normally present in preparations of extracellular enzymes as in the present case, namely, amyloglucosidase from Rhizopus delemar. Nevertheless, in order to use the present method to determine the activity of glucose-liberating enzymes, other than amyloglucosidases from micro-organisms, attention must be paid to this possible interference.

On the other hand, the absorbance of proteins can be overcome by adding them to the reference cell as well. Obviously, there will be a maximum limit for doing so, above which the amount of light reaching the detection is reduced, and therefore the noise of the recorder increases.

A lysozyme grade I solution of 5 $mg.ml^{-1}$ was found to be this limit when a Unicam SP 800 spectrophotometer was used. Therefore, protein preparations containing only 0.1% of amyloglucosidase may be used, but a lower percentage than this will not be measurable by this method.

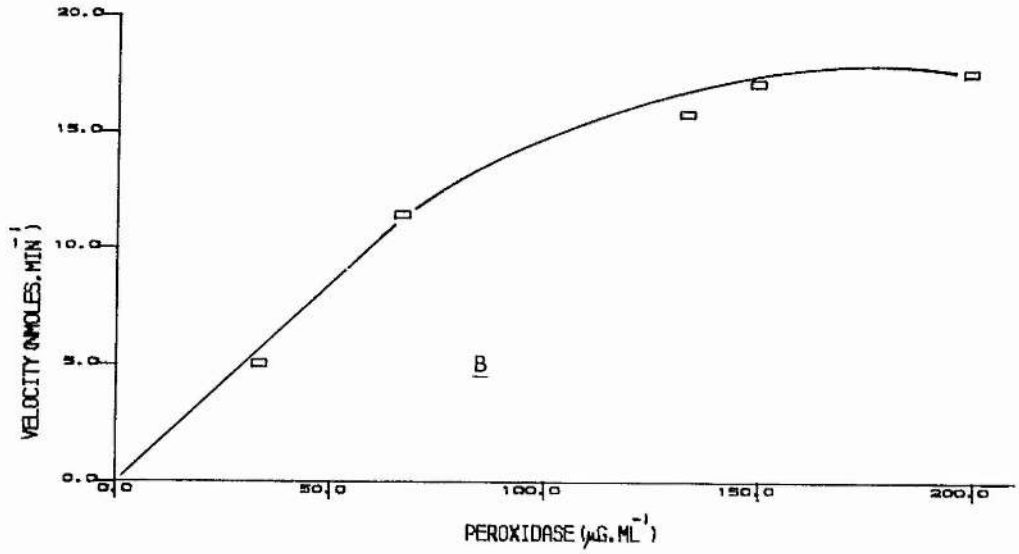
ii) Rate behaviour of the enzymic reaction:

The amounts of glucose oxidase and peroxidase required to ensure that the first reaction, releasing of glucose from α-1,4-glucans by amyloglucosidase, will be the rate limiting step in the oxidation of ascorbic acid, were found by the following two experiments:

1 - Increasing amounts of glucose oxidase were incubated in assay mixture composed of 50 μM L-ascorbic acid, 0.5% (w/v) amylose, 200 μg peroxidase, 100 μg amyloglucosidase and 50 mM phosphate/citrate buffer, pH 5.0 containing 70 mM sodium oxalate (volume = 3.00 ml, temperature = 40°); and the rate of ascorbic acid oxidation recorded. The plot of these rates against the amounts of glucose oxidase employed showed a hyperbola-shape (See Fig. 4.7.1.a). Therefore, quantities of glucose oxidase higher than 20 $\mu g.ml^{-1}$ will yield a negligible increase of the rate of ascorbic acid oxidation.

2 - The assay mixture of the last experiment was basically kept constant, using the obtained value of 20 $\mu g.ml^{-1}$ of glucose oxidase, whereas peroxidase varied and the plot of the rates of ascorbic acid oxidation against the quantities of peroxidase used showed again a hyperbola-shape (See Fig. 4.7.1.b). A minimum amount of 150 $\mu g.ml^{-1}$ peroxidase is therefore recommended.

The enzyme specific activities used were:



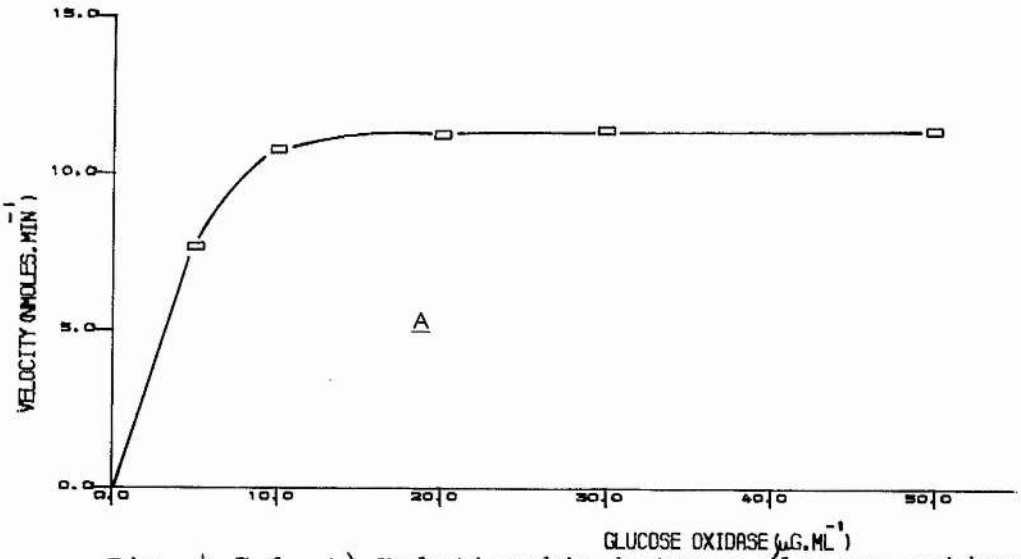


Fig. 4.7.1. A) Relationship between glucose oxidase concentration and the initial rate of ascorbic acid oxidation. Reaction mixture composed of 50 µM L-ascorbic acid, 0.5% (w/v) amylose, 200 µg peroxidase, 100 µg amyloglucosidase and 50 mM PC buffer containing 70 mM sodium oxalate, pH 5.0 (Vol. = 3.00 ml, T=40°)

B) Relationship between peroxidase concentration and the initial rate of ascorbic acid oxidation. Reaction mixture composed as above, except 20 µg/ml of glucose oxidase were used.

Amyloglucosidase - 0.528 units.mg protein^{-1}

Glucose oxidase - 210.0 units.mg protein^{-1}

Peroxidase - 3,354.21 units.mg protein^{-1}

Finally, by using these values of glucose oxidase and peroxidase, the activities of different amounts of amyloglucosidase were determined and a linear relationship between amyloglucosidase concentration and the velocity of ascorbic acid oxidation was found (See Fig. 4.7.2.). Furthermore, the specific activity of 0.528 units.mg protein^{-1} for the used enzyme preparation was in agreement with that determined by the glucose oxidase/oxygen electrode method.

The study of the rate behaviour of enzymic reactions such as presented here becomes very complicated when 3 reactions are involved. Nevertheless, the model can be simplified as follows:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D$$

Obviously, if the rate of conversion of B to C and C to D are greater than the rate of conversion of A to B, then the first reaction will be the rate-limiting step in the appearance of D.

Bergmeyer (74) has pointed out a requirement that k_2 should be at least 100 times greater than k_1. Moreover, he has shown an example of a 3-coupled enzymic reaction for the determination of myokinase, in which a ratio of k_3/k_2 of 30 proved satisfactory. It was also stated that the appearance of an induction period was consistent with a decrease in the ratio of k_3/k_2.

The enzyme activities employed in the present assay were:

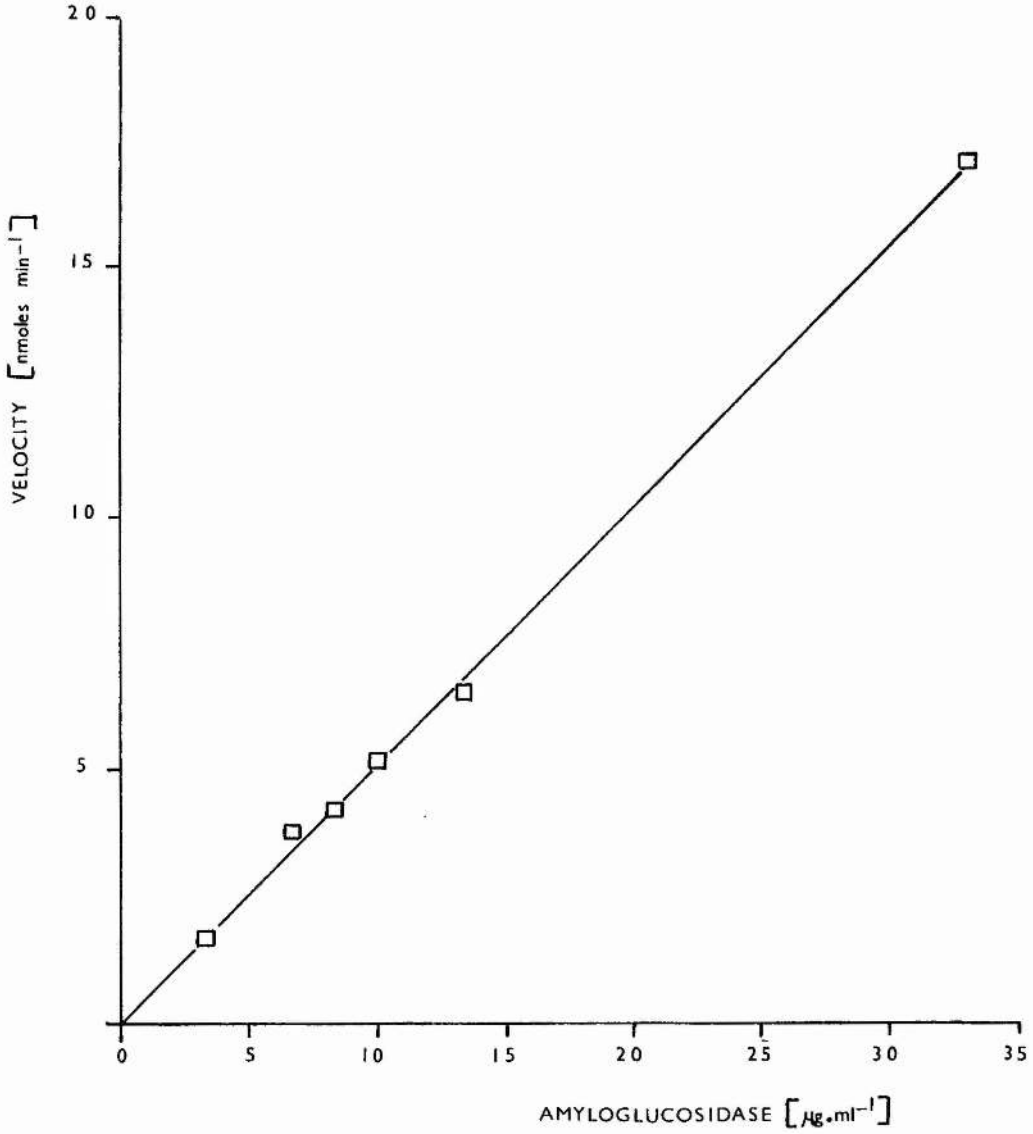


Fig. 4.7.2. Relationship between amyloglucosidase concentration and the initial rate of ascorbic acid oxidation. Reaction mixture composed of 50 µM L-ascorbic acid, 0.5% (w/v) amylose, 60 µg glucose oxidase, a 450 µg peroxidase, 50 mM PC buffer containing 70 mM sodium oxalate pH 5.0 (Vol.=3.00 ml, T=40°).

Amyloglucosidase - 0.018 units.ml^{-1}

Glucose osidase - 4.2 units.ml^{-1}

Peroxidase - 503.131 units.ml^{-1}

Therefore, these values of k_3 = 119.8 k_2 and k_2 = 233.3 k_1 are in agreement with those proposed by Bergmeyer (74). Even so, an induction period of about 1 min was found.

iii) Comparison against established procedure:

A comparison between this method and a conventional procedure of glucose-liberating enzyme assay was provided by the action of α-glucosidase (E.C. 3.2.1.20.) on p-nitrophenyl - α-D-glucoside.

This enzymic reaction can be represented as follows:

$$\text{p-Nitrophenyl-}\alpha\text{-D-glucoside} \xrightarrow{\alpha\text{-glucosidase}} \alpha\text{-D-glucose} + \text{p-nitrophenol}$$

The release of p-nitrophenol can be measured by spectrophotometry at 405 nm, whereas glucose can also be measured by spectrophotometry at 268 nm, since the method in discussion is employed. In this way, two values for the same hydrolysis rate will be found and a comparison can be made.

Reagents:

Enzymes:

α-Glucosidase - 1mg.ml^{-1} containing 3 units.ml^{-1}.

Glucose oxidase - 0.3mg.ml^{-1} containing 63 units.ml^{-1}

Peroxidase - 1mg.ml^{-1} containing 3.354 units.ml^{-1}

Substrates:

3.3 mM p-nitrophenyl-α-D-glucoside.

0.3 mM L-ascorbic acid

Buffer:

50 mM phosphate/citrate buffer, pH 6.8, containing 70 mM sodium oxalate.

Procedure:

The following were placed in cuvettes maintained at 40^{o}:

Reagents	Test	Control
α-glucosidase	10-30 µl*	as in test
p-Nitrophenyl-α-D-glucoside	40-100 µl*	-
L-Ascorbic acid	0.50 ml	-
Glucose oxidase	0.20 ml	0.20 ml
Peroxidase	0.45 ml	0.45 ml
Phosphate/citrate buffer	up to 3.00 ml	up to 3.00 ml

* - A micropipette was used.

The increase in extinction at 405 nm due to the release of p-nitrophenol and the decrease at 270 nm due to the oxidation of ascorbic acid were each recorded alternately for short periods (2 min.).

The molecular extinction coefficients for p-nitrophenol and ascorbic acid at pH 6.8 are 9.6×10^{3} $l.mol^{-1}.cm^{-1}$ and 12.6×10^{3} $l.mol^{-1}.cm^{-1}$, respectively.

By using these values the α-glucosidase activity can be calculated as follows:

$v_1^{*} = (\Delta E/min \times 3.0)/9.6$ at 405 nm

$v_2^{*} = (\Delta E/min \times 3.0)/12.6$ at 270 nm

Where Δ E/min stands for changing of extinction per minute.

* - expressed as $\mu moles.min^{-1}$

The results are presented in Table 4.7.1. As may be seen by inspection, v_1 and v_2 are the same (standard error of mean equal to 5.71%).

TABLE 4.7.1. - COMPARISON BETWEEN THE VALUES OBTAINED FOR THE HYDROLYSIS OF p-NITROPHENYL-α-D-GLUCOSIDE UPON α-GLUCOSIDASE CATALYSIS BY SPECTROPHOTOMETRY AT 400 nm (p-NITOPHENOL RELEASE) AND BY SPECTROPHOTOMETRY AT 268 nm (ASCORBIC ACID METHOD).

α-Glucosidase (μl)	PNPG (μl)	v_1^* (nmoles.min^{-1})	$v_2^@$ (nmoles.min^{-1})
10	100	12.187	11.904
10	100	12.000	12.000
30	50	16.250	20.000
30	40	14.062	13.571
30	60	18.750	18.809
15	100	18.750	14.762
20	100	22.812	18.333
30	100	35.937	38.095

* - Activity expressed in terms of p-Nitrophenol released.

@ - Activity expressed in terms of glucose released which was determined by the ascorbic acid method.

Despite the fact that α-glucosidase releases α-D-glucose and glucose oxidase catalyses the oxidation of only β-D-glucose (75), these results show that mutarotation is not a limiting factor in the use of the ascorbic acid method. Actually, mutarotation is not a consideration as far as the use of this procedure to determine amyloglucosidase activity is concerned, since this enzyme releases β-D-glucose (76).

iv) Presence of oxalate:

Sodium oxalate was introduced into the phosphate/citrate buffer to reduce further autoxidation of the ascorbic acid. The possibility of alteration of the amyloglucosidase activity by oxalate was investigated by comparing the kinetics obtained using the ascorbic acid method (in the presence of oxalate) and neocuproine method (in the absence of oxalate). These results are shown in Fig. 4.7.3. They can be summarized as follows:

Kinetic parameters	Ascorbic acid method	Neocuproine method
Km(μM)	15.72 $\pm$ 0.89	15.78 $\pm$ 1.39
V(nmoles.min^{-1})	39.34 $\pm$ 1.43	5.44 $\pm$ 0.19
k_{+2}(s^{-1})	15	16.5
(E)(M)*	1.456×10^{-8}	5.5×10^{-9}

* - Enzyme concentration used (mol.wt. = 100,000 (9)).

These results show an agreement between both methods, therefore the addition of oxalate to prevent autoxidation of ascorbic acid does not affect amyloglucosidase activity.

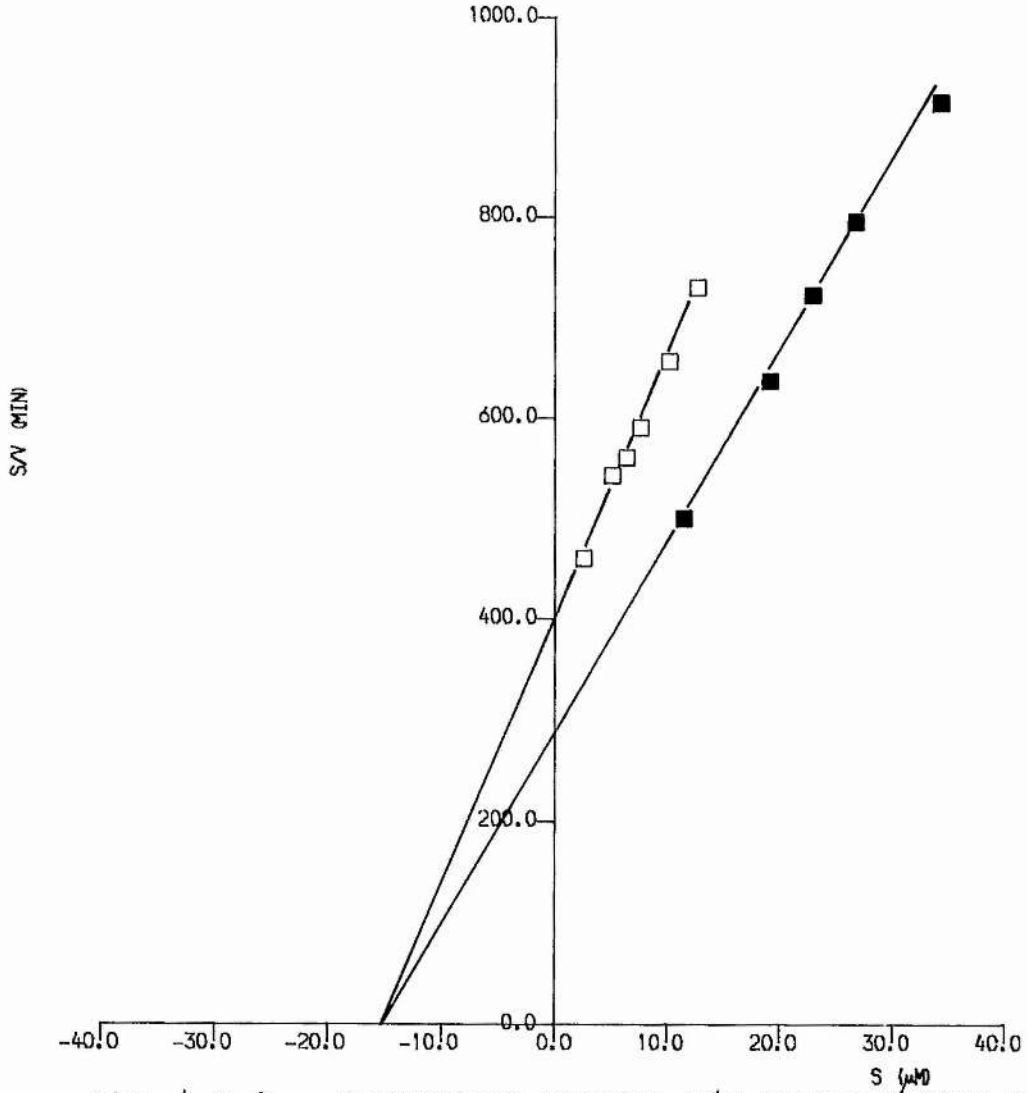


Fig. 4.7.3. Comparison between s/v versus s plot for amyloglucosidase catalysed hydrolysis of amylose obtained using the ascorbic acid method (□) and the neocuproine method (■) pH 5.0, T = 40°. Enzyme concentration in the ascorbic acid method and neocuproine method were 0.15 nM and 5.5 nM, respectively.

4.8. - STUDIES ON IMMOBILISED AMYLOGLUCOSIDASE.

Immobilised amyloglucosidase derivatives have been prepared by reacting amyloglucosidase with CM-cellulose hydrazide and p-aminobenzylcellulose (PAB-cellulose). The values of bound protein and enzyme activity were as follows:

Method	Bound protein* (mg/100mg of derivative)	Enzyme Activity (units/mg bound prot.)	Activity retained (%)
Chemical coupling to CM-cellulose hydrazide	17.5	0.162	34.2
Chemical coupling to PABC	4.5	0.013	2.74

* - The protein chemically coupled was determined as the amount of protein in the reaction mixture that was not found in the filtrate and wash solutions.

The results obtained (bound protein, enzyme activity and activity retained) by coupled amyloglucosidase onto CM-cellulose hydrazide were lower than those originally reported by Christison (44).

Fig. 4.8.1 presents s/v versus s plot of CM-cellulose-amyloglucosidase derivative action on soluble starch compared with that for the native enzyme. The value of Km (0.060 ± 0.005 % (w/v)) and V(162.4 ± 6.5 mmoles.min.$^{-1}$ mg protein^{-1}) for this immobilised amyloglucosidase were different from those found for the native enzyme (0.0068 ± 0.0008 % (w/v) and 475.0 ± 23.7 mmoles.min.$^{-1}$ mg protein^{-1}). Although such immobilisation procedure involves the prosthetic group of amyloglucosidase, which is reported to have no apparent role in the catalytic function of the enzyme (19) an approximately 9-fold increase

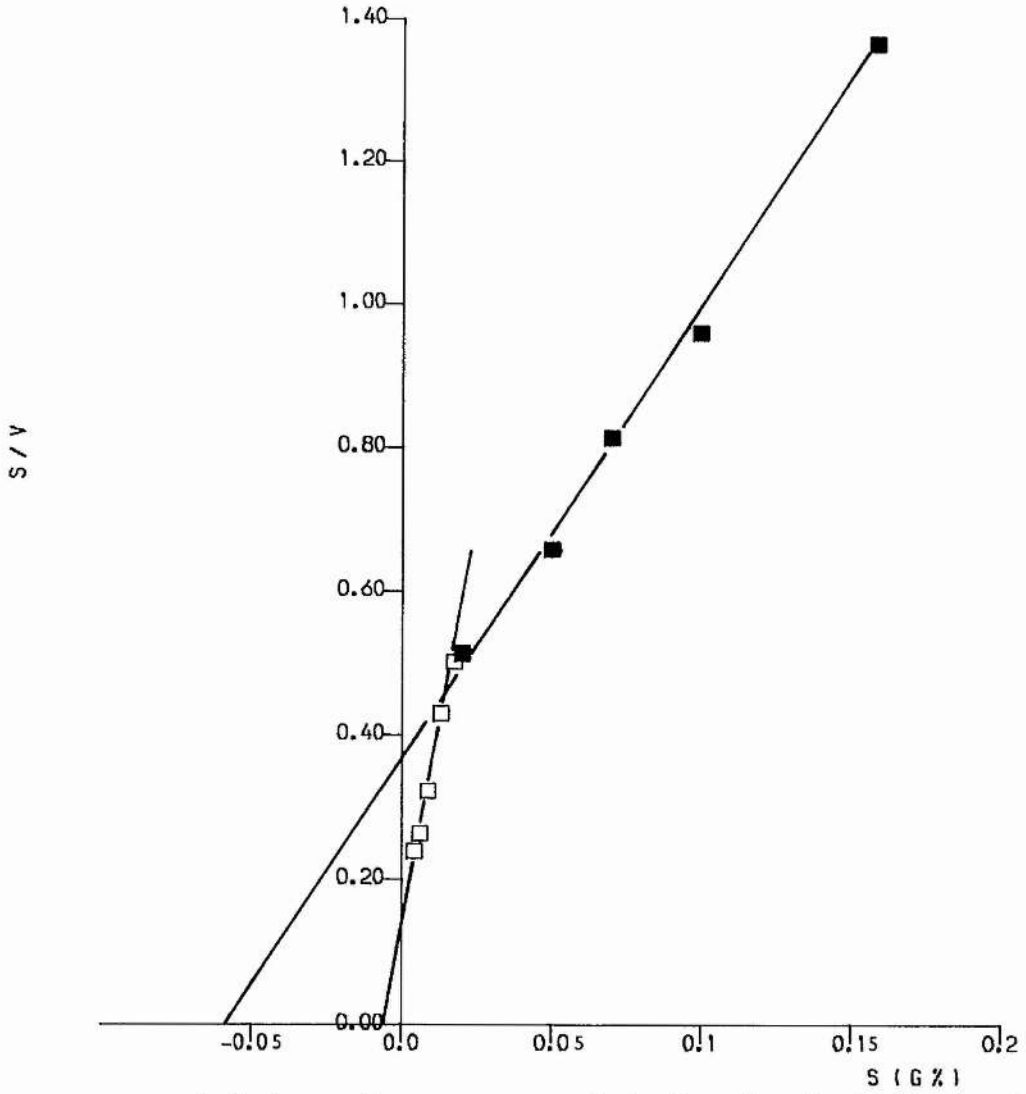


Fig. 4.8.1. s/v versus s plot for the hydrolysis of soluble starch upon free amyloglucosidase (□) catalysis and CM-cellulose amyloglucosidase (■) catalysis; pH 5.0, T=40°. s/v is expressed in 10^{3} x % (w/v).nmoles^{-1}.min.

in Km and an approximately 3-fold decrease in Vm were observed for the immobilised enzyme compared with the free enzyme. Since substrate of larger molecular weight was employed film diffusion resistence effects would explain this increase in Km. The observed decrease in V is well documented and typical of preparations of immobilised enzyme (39).

Fig. 4.8.2. shows s/v versus s plot for the action on soluble starch of amyloglucosidase covalently coupled to PAB-cellulose. The observed changes in Km (0.233 ± 0.016 % (w/v)) and V (13.028 ± 0.344 mmoles.min.$^{-1}$ mg protein^{-1}) are rather greater than those discussed above for CM-cellulose-amyloglucosidase. PAB-cellulose has been recognised as a poor support material (77) usually yielding low retention of enzymatic activity.

Fig. 4.8.3. shows the pH profile for CM-cellulose-amyloglucosidase and of the free enzyme. An alkaline shift observed for the CM-cellulose-amyloglucosidase is in agreement with information collected so far in immobilised enzyme studies since a negatively charged support is employed in this case (54).

Fig. 4.8.4. presents the thermal stability of CM-cellulose-amyloglucosidase compared with that of the native enzyme. A more stable enzyme preparation was found after immobilisation. It is interesting to note that the amyloglucosidase stability reduced during the oxidation of the prosthetic group (19) (the step preceding immobilisation) is recovered and enhanced after this oxidised carbohydrate prosthetic group becomes involved in the covalent linkage to the support.

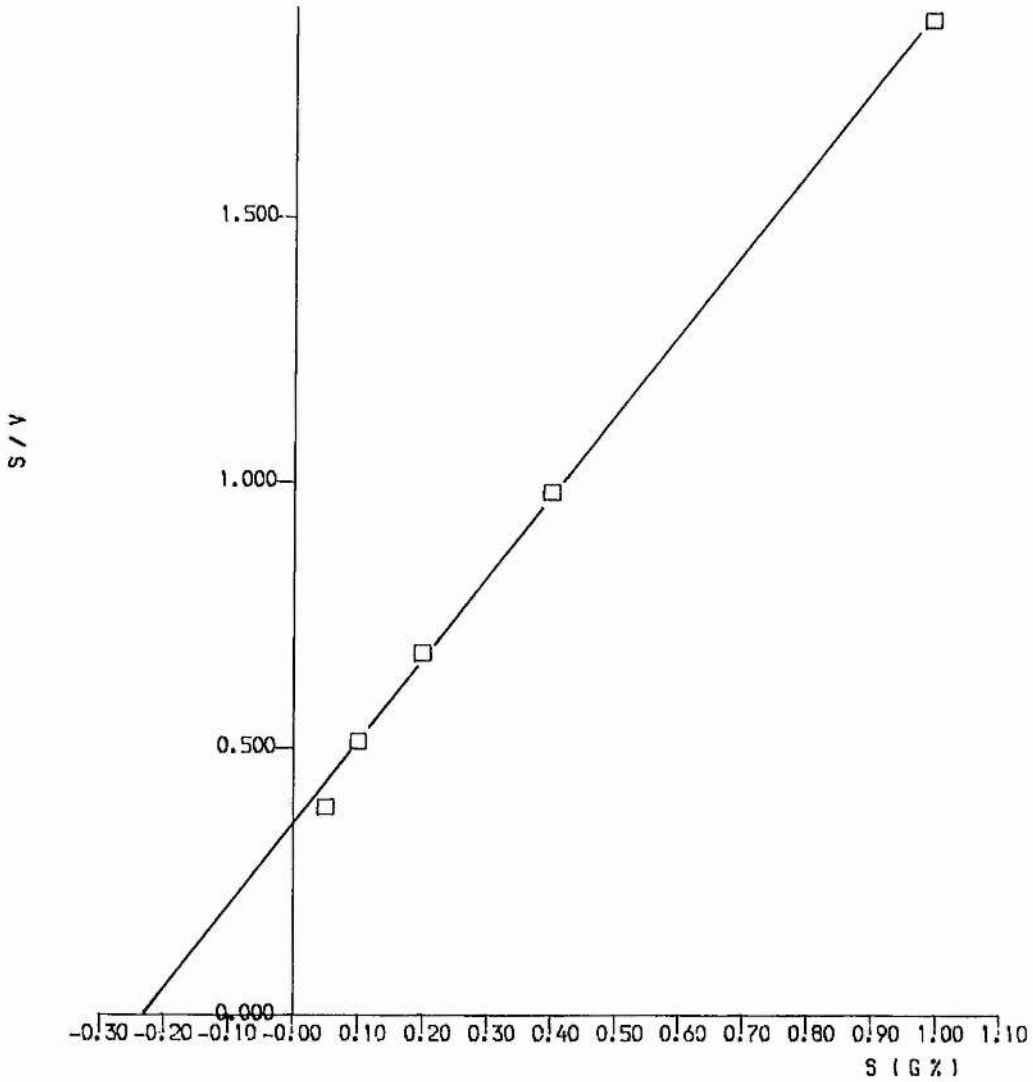


Fig. 4.8.2. s/v versus s plot for the hydrolysis of soluble starch upon PAB-cellulose-amyloglucosidase catalysis. pH 5.0, T = 40^{o}. s/v is expressed in 10^{3} x % (w/v).nmoles^{-1}. min.

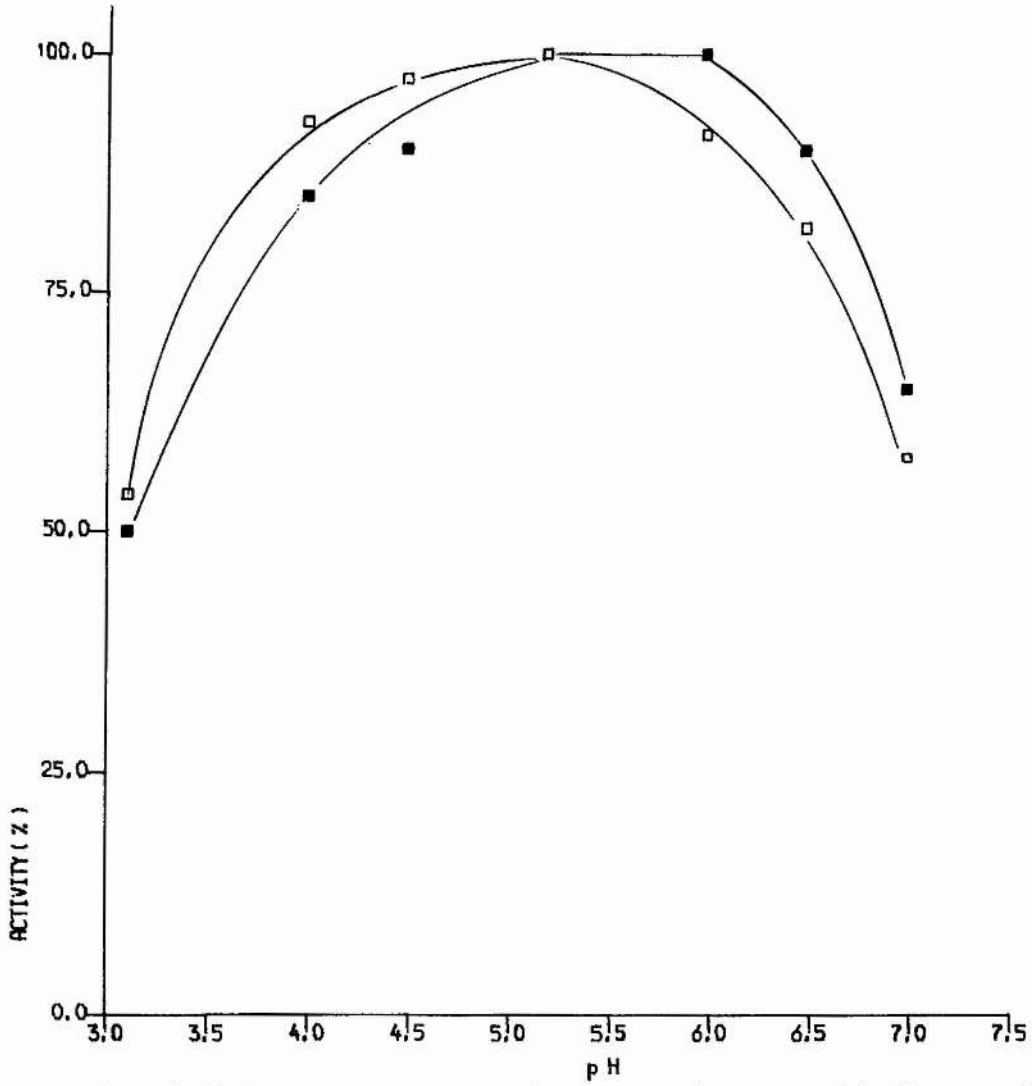


Fig. 4.8.3. - Comparison between the pH activity profiles of free amyloglucosidase (□) and CM-cellulose-amyloglucosidase (■).

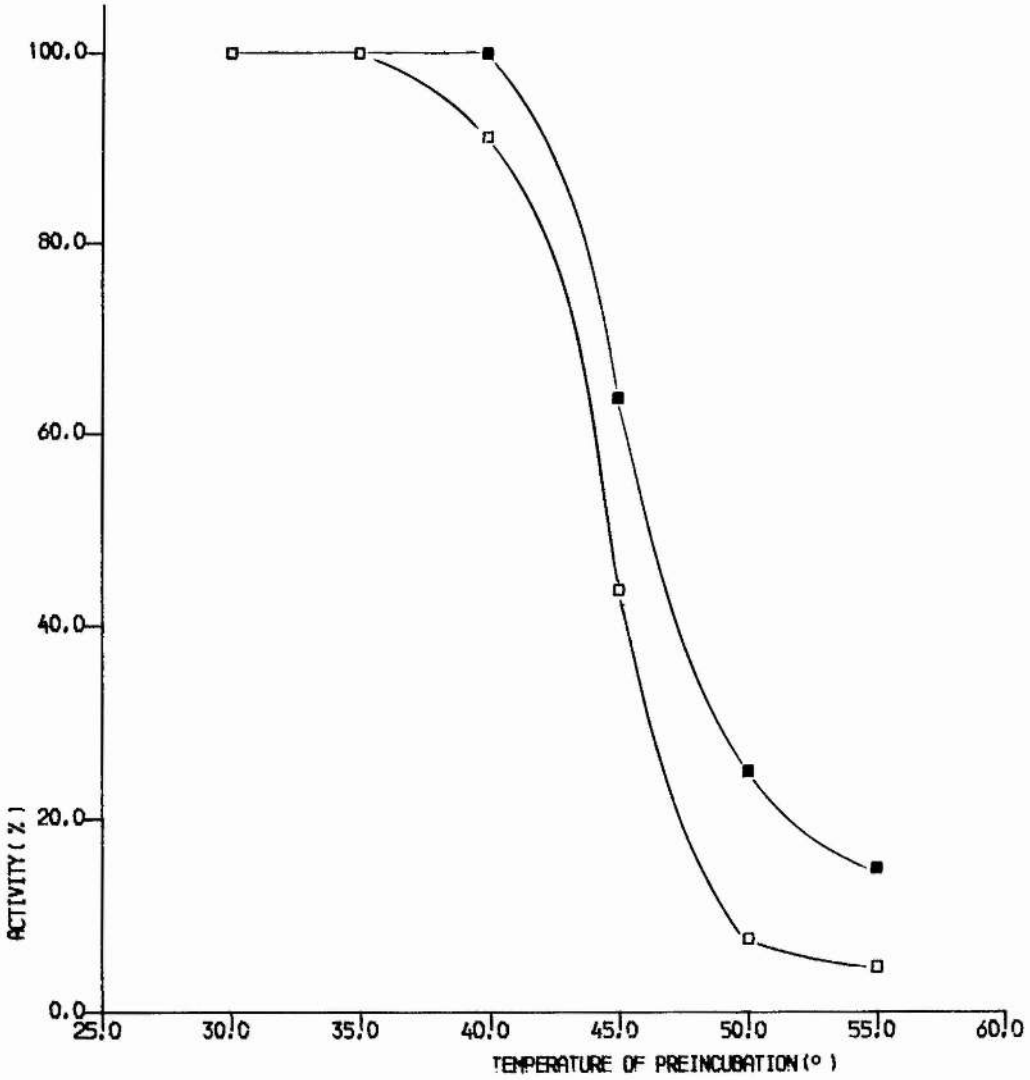


Fig. 4.8.4. - Comparison between the thermal stabilities of free amyloglucosidase (□) and CM-cellulose-amyloglucosidase (■).

5. - SUMMARY.

1 - Amyloglucosidase from Rhizopus delemar has been purified by ammonium sulphate fractionation; ethanolic precipitation; chromatography on DEAE-cellulose, DEAE-sephadex A-50 and Sephadex G-50/Ambolite IRC-50. Ethanolic precipitation and chromatography on DEAE-cellulose by stepwise elution have been proved to be the most simple and rapid procedure to remove traces of α-amylase from amyloglucosidase preparations.

2 - Amyloglucosidase free of traces of α-amylase has been shown to be incapable of releasing coloured products from Remazol Brilliant Blue derivatives of α-1,4-glucans.

3 - The presence of α-amylase activity in amyloglucosidase preparations has been clearly shown to alter the apparent kinetic parameters of amyloglucosidase.

4 - A theory has been developed to describe the action of amyloglucosidase on partially oxidised amyloses. Experiments have been carried out to test this theory.

5 - A direct recording method of assaying amyloglucosidase has been developed involving ascorbic acid as the oxygen acceptor in a glucose oxidase/peroxidase assay system for glucose. This method permits amyloglucosidase activity to be followed by ultraviolet spectrophotometry. The merits and limitations of this procedure have been discussed.

6 - An immobilised cellulose derivative of amyloglucosidase in which the prosthetic group is involved in the covalent linkage between the enzyme and the support has been investigated and compared with the soluble enzyme.

APPENDIX

DATA PROCESSING:

Throughout this project much data has been displayed graphically by plotting each data point on a two-dimensional x, y plane. These points were usually scattered on the grid so that statistical treatment was required wherever possible. The majority of these correlations were linear ones and only these were statistically analysed. The approach consisted of finding the regression line and its confidence limits. Statistical methods for fitting enzyme kinetic data were also used to obtain more accurate estimates and the necessary measure of precision.

The theory behind these statistical estimations is presented in this chapter.

Although the theory of regression line estimation is well documented, it is included here for the sake of comprehension of the theory of statistical treatment of kinetic data.

i) THE ESTIMATION OF A REGRESSION LINE AND ITS CONFIDENCE INTERVAL.

A regression line is the straight line in which the mean of y-values for each x-value lies (linear correlation).

Firstly, let $\tilde{y}$ be the true value of y (dependent variable) at any given value of x (independent variable). Then the equation of the regression line will be

$$\tilde{y} = a + bx$$

where a is the intercept on the y-axis at $x = 0$, and b is the slope (regression coefficient).

Therefore, y values will scatter below and above the regression line and such deviations will be defined by $y - \tilde{y}$. The sum of these squared deviations $\sum(y - \tilde{y})^2$ must be as small as possible. Hence, this method of estimating a regression line is called the "least square" method.

In order to find the values of a and b which will minimize

the sum of squared y-deviations from the line, we set the value of the deviation at a and b is equal to zero. Thus we denote:

$$\frac{dQ}{da} = 0 \quad (1) \quad \text{and} \quad \frac{dQ}{db} = 0 \quad (2)$$

where $Q = \sum (y-\bar{y})^2$ (3)

Introducing $\tilde{y} = a + bx$ (4)

$$Q = \sum (y - a - bx)^2 \quad (5) \text{ or } Q = (\sum y - Na - b\sum x) \quad (6)$$

Where N stands for the number of experimental points. Solving equation 1 by partial differentiation yields,

$$0 = 2 (\sum y - Na - b\sum x) \quad (7)$$

Then

$$a = \frac{\sum y - b\sum x}{N} \quad (8)$$

$$a = \bar{y} - b\bar{x} \quad (9)$$

Partial differentiation with respect to b gives

$$0 = 2(\sum xy - a\sum x - b\sum x^2) \quad (10)$$

By introducing the value of a (equation 9) in the above equation, then b can be given by

$$b = \frac{\sum xy - \bar{y}\sum x}{\sum x^2 - \bar{x}\sum x} \quad (11)$$

By multiplying both the numerator and the denominator by N,

$$b = \frac{N\sum xy - \sum y\sum x}{N\sum x^2 - (\sum x)^2} \quad (12)$$

By using these values of a (equation 9) and b (equation 12) the regression line can easily be found.

The establishment of a confidence interval for the regression line was made by finding the interval for $\tilde{y}$ at the central region of the regression line (mean of x, $\bar{x}$).

The standard error of a mean is obtained by dividing the square root of the error variance (standard deviation) by the square root of the number of experimental points. The error

variance is computed by the equation*

$$S_{y.x}^2 = \frac{1}{N-2}\left[\sum y^2 - \frac{(\sum y)^2}{N} - b\left(\sum xy - \frac{\sum x . \sum y}{N}\right)\right] \quad (13)$$

The standard error of a mean will be given by

$$S_{\bar{y}} = \frac{S_{y.x}}{\sqrt{N}} \quad (14)$$

* - For details see Reference no. 78.

Finally, the confidence interval for the regression line will be:

$$y = \bar{y} \pm t.S_{\bar{y}}$$

Where t has (N-2) degree of freedom and its critical values can be found in available tables (78).

A FORTRAN program was written for estimating the regressi line by the least square method and for calculating the confidence interval.

Symbols used in this program:

X and Y:- Independent and dependent variables.

SX, SY, SXY, SX2 and SY2:- Summation of X, Y, XY, X^2 and Y^2 respectively.

YM :- The mean of Y variables.

B and A :- Regression coefficient and intercept calculated according to equation 12 and 9, respectively.

C :- True value of Y (best fit) given by equation 4.

V :- Variance given by equation 13.

SD :- Standard deviation given by the square root of variance.

SE :- Standard error given by equation 14.

P :- Degree of freedom.

T :- Critical value of t(Student's t-test).

JJ :- Number of data sets processed.

NP :- Number of data values in a given data set.

I :- Counting indice for X and Y arrays and for a DO loop in the program.

Program:

```
C       PROGRAM BEST FIT AND CONFIDENCE INTERVAL
        DIMENSION X(200),Y(200)
        PRINT 1
     1  FORMAT(75H CALCULATION OF BEST FIT(LEAST SQUARES METHOD) AND ITS
       XCONFIDENCE INTERVAL///)
     2  FORMAT(I3,17X,48H ANYTHING HERE WILL BE PRINTED DURING OUTPUT    )
     3  FORMAT(2F10.4)
        JJ = 0
    12  READ 2,NP
        IF(NP) 11,11,4
     4  SX = 0
        SY = 0
        SXY= 0
        SX2= 0
        SY2 = 0
        P = NP-2
        JJ = JJ + 1
        PRINT 2,JJ
        PRINT 14
    14  FORMAT(41H DATA      X VALUES              Y VALUES)
        DO 5 I = 1,NP
        READ 3,X(I),Y(I)
        SX = SX + X(I)
        SY = SY + Y(I)
        SXY = SXY + X(I)*Y(I)
        SX2 = SX2 + X(I)*X(I)
        SY2 = SY2 + Y(I)*Y(I)
     5  PRINT 15,X(I),Y(I)
    15  FORMAT(10X,F10.5,13X,F10.5)
        YM = SY/NP
        B = ((NP*SXY)-(SX*SY))/((NP*SX2)-(SX**2))
        A = (SY-B*SX)/NP
        C = A + B*X(NP)
        PRINT 6,A
        PRINT 7,C
     6  FORMAT(37H THE INTERCEPT ON Y-AXIS AT X=0 IS    ,F10.5/)
     7  FORMAT(45H FOR THE HIGHEST VALUE OF X,THE BEST FIT IS  ,F10.5/)
        V = SY2-(SY**2/NP)-B*(SXY-(SX*SY/NP))
        V = V/P
```

```
      SD  = SQRT(V)
      SE = SD/SQRT(FLOAT(NP))
      PRINT 9,SE
      PRINT 10,YM,SE
    9 FORMAT(35H THE STANDARD ERROR OF Y-MEAN IS    ,F10.5/)
   10 FORMAT(39H THE CONFIDENCE INTERVAL AT X-MEAN IS    ,F10.5,10H + OR
     X- T.,F10.5///)
      GO TO 12
   11 PRINT 13,JJ
   13 FORMAT(23H PROGRAM COMPLETED FOR I4,6H LINES)
      STOP
      END
```

The input required by this program consists of a title card with the number of data points placed in columns 1-3 and any desired title (data set title) in columns 21-69, and a data card for each point with X and Y values placed in columns 1-10 and 11-20, respectively. A great number of regression analyses can be calculated by following these procedures provided that the number of total points does not exceed the maximum established in the DIMENSION statement. A blank card must be placed at the end of the data deck to provide an end to the program.

The functions of the various parts of the program are as follows:

The DIMENSION statement assigns a memory location to X and Y arrays.
200 data values were the maximum number of rows for X and Y, but it can be increased for any required number.

PRINT 1 provides a main title to the output. Statement 12 reads the first card from the input according to format 2. If the number of data points (NP) is blank the following IF STATEMENT will provide for the termination of the program. If NP is positive STATEMENT 4 and subsequent statements will set all summations equal to zero, calculate the degree of freedom and print the data set title read in STATEMENT 12 as well as a title head to the output (Format 14). By increasing the value of JJ by one the calculations of the data sets will be numbered during the output.

DO loop specifies the values of X and Y to be read according to Format 3, calculates the summations and prints the values of X and Y. With the results of the summations, A and B are calculated and the best fit for the highest value of X (C) is also calculated. Then, the intercept A and the best fit C are printed out. These two points are sufficient for tracing the regression line. Finally, the variance, standard deviation and standard error are calculated and the values of standard error and the confidence interval at the mean value of X are printed out. The STATEMENT GO TO 12 either initializes regression analyses for another data set or terminates the calculations if a blank card follows.

*

$$a = \bar{y} - b\bar{x}$$

or $\bar{y} = a + b\bar{x}$

relates to the equation

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{1}{S}$$

ii) STATISTICAL ESTIMATION OF ENZYME KINETICS.

The statistical treatment of enzyme kinetic data was introduced by Johansen & Lumry (79) and by Wilkinson (80) and has been extended to more complex rate equations by Cleland (81).

Fitting of data to linear forms of Michaelis-Menten equation, $1/v = 1/V + Km/(V.S)$ (1) or $S/v = Km/V + S/V$ (2) can be done by the least square method. Weighting of individual data points is advisable and Wilkinson (80) has used a weighted regression procedure to obtain provisional estimates of Km and V. Fine adjustments were provided by fitting the Michaelis-Menten function directly in the hyperbolic form. Wilkinson's method is as follows:

Calculation of provisional estimates of Km and V:

As seen earlier, the regression coefficient, the slope of the regression line, was found to be:

$$b = \frac{\sum xy - \bar{y}\sum x}{\sum x^2 - \bar{x}\sum x} \qquad (3)$$

and the intercept on the y-axis, at $x = 0$,

$$a = \bar{y} - b\bar{x} \quad (4)^{*}$$ See opposite page

Since the weighted means are:

$$\bar{x} = \frac{\sum wx}{\sum w} \quad (5) \text{ and } \bar{y} = \frac{\sum wy}{\sum w} \quad (6)$$

the weighted equations 3 and 4 will be

$$b = \frac{\sum wxy - \sum wx.\sum wy/\sum w}{\sum wx^2 - (\sum wx)^2/\sum w} \quad (7) \quad \text{and} \quad a = \frac{\sum wx}{\sum w} - b\frac{\sum wx}{\sum w} \quad (8)$$

The values of x, y, a and b for the present case can easily be found by using the linear form of Michaelis-Menten equations, for instance, equation 1. In cases where rates are measured at a constant enzyme concentration, the variance will be essentially constant, so that the weighting (w) factors are simply v^4. Then equations 7 and 8 become

$$b = \frac{\sum v^3/s - (\sum v^4/s \sum v^3)/\sum v^4}{\sum v^4/s^2 - (\sum v^4/s)^2/\sum v^4} \quad (9) \text{ and } a = \frac{\sum v^3}{\sum v^4} - b\,\frac{\sum v^4/s}{\sum v^4} \quad (10)$$

Using the author's notations,

$\alpha = \sum v^3$, $\beta = \sum v^4$, $\gamma = \sum v^3/s$, $\delta = \sum v^4/s$ and $\epsilon = \sum v^4/s^2$

the equations 9 and 10 simplifies to

$$b = \frac{\beta\gamma - \delta\alpha}{\epsilon\beta - \delta^2} \quad (11) \quad \text{and} \quad a = \frac{\alpha - b\delta}{\beta} \quad (12)$$

Replacing a by 1/V in equation 12 and introducing the value of b, gives

$$V = \frac{\epsilon\beta - \delta^2}{\alpha\epsilon - \gamma\delta} \quad (13)$$

and since $b = Km/V$, Km can be calculated by using the values of b and V found in equations 11 and 13, respectively. So,

$$Km = \frac{\gamma\beta - \delta\alpha}{\alpha\epsilon - \gamma\delta} \quad (14)$$

Thus the provisional estimates of V and Km are found.

Calculation of fine estimates of V and Km:

The Michaelis-Menten equation can be expressed approximately by the linear part of a Taylor expansion,

$$v \approx \frac{V}{V^o}\left[\frac{S.V^o}{S+Km} + (Km^o - Km).\ \frac{S.V^o}{(S+Km)^2}\right] \quad (15)$$

Where Km, V and Km^o, V^o stand for fine and provisional parameters, respectively.

Equation 15 simplifies to

$$v \approx b_1.f(S) + b_2.f'K(S) \quad (16)$$

where $b_1 = V/V^o$, $b_2 = b_1\,(Km - Km^o)$, f(S) is the provisional fit as described previously and f'K(S) is the first derivative of f(S) with respect to Km.

Equation 16 is linear and depends on two determining variables, such as:

$$y = a + b_1 x_1 + b_2 x_2 \quad (17)$$

Using the same treatment described in item 1 of this appendix (The estimation of a regression line and its confidence interval) two equations are obtained for the regression coefficients b_1 and b_2:

$$\alpha b_1 + \gamma b_2 = \delta \quad (18)$$

$$\gamma b_1 + \beta b_2 = \epsilon \quad (19)$$

in which the following Wilkinson's notations were introduced:

$$\sum x_1^2 = \alpha$$

$$\sum x_2^2 = \beta$$

$$\sum x_1 . x_2 = \gamma$$

$$\sum x_1 . y = \delta$$

$$\sum x_2 . y = \epsilon$$

Solving equations 18 and 19, the values of b_1 and b_2 are given by

$$b_2 = \frac{\epsilon\alpha - \delta\gamma}{\alpha\beta - \gamma^2} \quad (20) \text{ and } b_1 = \frac{\beta\delta - \gamma\epsilon}{\alpha\beta - \gamma^2} \quad (21)$$

and finally, $V = b_1 v^o$ (22) and $Km = Km^o + b_2/b_1$ (23).

These values can be used as provisional estimates and the last fitting process repeated to obtain finer adjustments, until $(Km - Km^o)$ is reduced nearly to zero (iterative method). The error variance in the present case is computed by the equation:

$$s^2 = \frac{\sum v^2 - b_1\delta - b_2\epsilon}{N - 2} \quad (24)$$

The standard errors for Km and V are derived from those for b_1

and b_2. Since the standard errors of b_1 and b_2 are given by

$$S.E.(b_1) = \sqrt{\frac{\beta}{\alpha\beta - \gamma^2}} \cdot S^* \quad (25) \qquad S.E.(b_2) = \sqrt{\frac{\alpha}{\alpha\beta - \gamma^2}} \cdot S^* \quad (26)$$

* - Square root of equation 24.

then the standard errors of Km and V, by application of the equations 23 and 22, will be

$$S.E.(Km) = S.E.(b_2)/b_1 \quad (27) \qquad S.E.(V) = V^o.S.E.(b_1) \quad (28)$$

For details see Reference no. 80.

A FORTRAN program was written for fitting data to Michaelis-Monten equation. For the sake of comprehension, a modified Cleland's program was used rather than his original program (81), since the latter was based upon a different approach. However, the results obtained by both programs were the same.

Symbols used in this program:

V(1),V(2),etc - Experimental velocities.

A(1),A(2),etc - Corresponding substrate concentrations.

W(1),W(2),etc - Weighting factors for velocities.

S(1,1),S(1,2),etc - The array in which , , , etc are solved.

Q(1),Q(2), etc - The array used to calculate the S array.

SV2 - Summation of V^2.

JJ - Number of data sets processed.

NP - Number of data cards following title card.

M - With M = 1 the matrix solution subroutine uses statements 15 and 16 to make the provisional estimates of Km and V. With M = 2, statements 17 and 18 are used to make the fine estimates of Km and V.

CK - Km

VM - Maximum velocity

NT - Number of iterations. This is set at 3 here.

DD - Denominators used to simplify arithmetical operations.

S2 - Experimental variance.

S1 - Square root of experimental variance (Sigma).

SECK,SEV - Standard error of the estimates of Km and V, respectively.

I,J,K - Counting indices for various DO-loops in the program

Program:

```
C       PROGRAM HYPER                                          <— arrays
        DIMENSION V(100),A(100),W(100),S(2,3),Q(3)
        PRINT 100
  100 FORMAT(35H FIT TO HYPERBOLA  V = VMAX*A/(K+A)///)
   11 FORMAT(I3,17X,46H ANYTHING HERE WILL BE PRINTED OUT DURING OUTPUT )
    1 FORMAT(3F10.5)
        JJ = 0
   14 READ 11,NP
        IF(NP) 99,99,12
   12 M = 1
        SV2 = 0
        P = NP - 2
        JJ = JJ + 1
        PRINT 11,JJ
        PRINT 37
   37 FORMAT(5HODATA,5X,8HVELOCITY,5X,9HSUBSTRATE,5X,6HWEIGHT)
        GO TO 2
   15 READ 1,V(I),A(I),W(I)
        PRINT 38,V(I),A(I),W(I)
   38 FORMAT(10X,F10.5,3X,F10.5,2X,F10.5)
        IF(W(I)) 19,19,20
   19 W(I) = 1
   20 Q(1) = V(I)**2/A(I)
        Q(2) = V(I)**2
        Q(3) = V(I)
        SV2 = SV2 + Q(2)
        GO TO 13
   16 DD = (S(2,3)*S(1,1) - S(1,3)*S(2,1))
        CK = ((S(2,2)*S(1,3)) - (S(2,3)*S(2,1)))/DD
```

```
      NT = 0
      M = 2
      GO TO 2
   17 D = CK + A(I)
      Q(1) = A(I)/D
      Q(2) = -Q(1)/D
      Q(3) = V(I)
      GO TO 13
   18 DD = (S(1,1)*S(2,2) - S(2,1)**2)
      B1 = ((S(2,2)*S(1,3)) - (S(2,1)*S(2,3)))/DD
      B2 = ((S(1,1)*S(2,3)) - (S(2,1)*S(1,3)))/DD
      CK = CK + B2/B1
      VM = B1
      NT = NT + 1
      IF(NT-3)2,21,21
   21 S2 = SV2 - B1*S(1,3) - B2*S(2,3)
      S2 = S2/P
      S1 = SQRT(S2)
      SECK = (S1/B1)*SQRT(S(1,1)/DD)
      SEV = S1*SQRT(S(2,2)/DD)
      PRINT 30,CK,SECK
      PRINT 31,VM,SEV
      PRINT 35,S2,S1
   30 FORMAT(7HOK     = F12.6,13H S.E.(K)     = F11.6)
   31 FORMAT(7H V     = F12.6,13H S.E.(V)     = F11.6)
   35 FORMAT(12H VARIANCE = E14.5,10H  SIGMA = F12.7//)
      GO TO 14
C     MATRIX SOLUTION SUBROUTINE
    2 DO 3 J = 1,3
      DO 3 K = 1,2
    3 B(K,J) = 0
```

```
      DO 4 I = 1,NP
      GO TO(15,17),M
   13 DO 4 J = 1,3
      DO 4 K = 1,2
    4 S(K,J) = S(K,J) + Q(K)*Q(J)*W(I)
      GO TO(16,18),M
   36 FORMAT(23H PROGRAM COMPLETED FOR I4,6H LINES)
   99 PRINT 36,JJ
      STOP
      END
```

Statement 14 and 15 formulate the input for this program according to format 11 and 1. Therefore, as in the previous program, this must be an initial card containing the number of data points placed in columns 1-3 and any desired title in columns 21-69. Then follows a data card for each point with velocity, substrate and weight placed in column 1-10, 11-20 and 21-30, respectively. By doing this, as many data sets as wanted can be used. A blank card must be added to the end of the data deck.

Statement DIMENSION specifies a memory location to V,A, W,Q and SS arrays. The statement PRINT 100 gives the output a main title. After JJ is set equal to zero, statement 14 causes the data points and the data set title to be read. If the former (NP) is blank, then the statement 99 follows, which ends the program with the sentence written in format 36. If NP is a positive number, then M is set equal to 1, SV2 is set equal to zero, the degree of freedom is calculated, JJ is increased by 1 and the data set title as well as the head of the next output are printed out. Statement GO TO 2 initializes the matrix solution subroutine which begins setting all the S arrays equal to zero. Then the DO loop 4 calculates the S array using the values of V,A and W read in statement 15 (M=1). PRINT 38 causes all the data points to be printed during output. If no weight is provided during input, then W will be set equal to 1 (IF STATEMENT and statement 19).

The S array can also be set out as follows:

S array	equivalent*	Wilkinson's notation
S(1,1)	$\sum v^4/s^2$	ϵ
S(2,1)	$\sum v^4/s$	δ
S(1,2)	$\sum v^4/s$	δ
S(2,2)	$\sum v^4$	β
S(1,3)	$\sum v^3/s$	γ
S(2,3)	$\sum v^3$	α

* - W=1

Since M = 1, the final statement in the matrix solution subroutine transfers the control to statement 16 and then the provisional Km is calculated according to equation 14.

Following the provisional estimations, the fine estimation are started by making NT=0 and M=2. The latter statement causes the matrix solution subroutine to set the S array in terms of Q's defined after statement 17. According to Wilkinson's notations Q(1) = f, Q(2) = f' and Q(3) = v. Therefore the S array will have another interpretation from that established previously. Here it will be:

S array	Equivalent*	Wilkinson's notatio
S(1,1)	$\sum f^2$	α
S(2,1)	$\sum f'.f$	γ
S(1,2)	$\sum f.f'$	γ
S(2,2)	$\sum f'^2$	β
S(1,3)	$\sum f.v$	δ
S(2,3)	$\sum f'.v$	ϵ

* - W=1

Statement GO TO 18 causes b_1, b_2 and Km to be worked out according to equations 21,20 and 23, respectively; NT is increased by 1 and statement IF(NT-3) causes all the fine estimations to be recalculated three times - Iterative method.

Afterwards, the experimental variance and its square root are calculated, as are the standard errors of Km (equation

27) and V (equation 28). Finally, these values are printed out and the statement GO TO 14 restarts data processing for another data set. If none is available the blank card for NP will end the program according to statement IF(NP).

It should be noted that the provisional estimate of V is n calculated nor its value employed during the fine estimation calculations. The lack of necessity for these calculations is based upon the observation that V^o (provisional V) can be cancelled in equation 15, then V will be given by only $b_1.V^o$ is also eliminated in the calculation of the standard error of V.

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